Universidade de Coimbra

Faculdade de Ciências e Tecnologia

Departamento de Ciências da Vida

Translational regulation mediated by internal ribosome entry sites of the *MTOR* and *Δ133P53* human transcripts

Ana Lúcia Marques Ramos

Dissertação submetida para obtenção do grau de Doutor em Biociências, especialidade em Biologia Molecular e Celular

Dissertation submitted for the degree of Doctor of Philosophy in Biosciences, specialization in Molecular and Cellular Biology

Orientadores (*Supervisors*):
Doutora Luísa Romão, Ph.D. (Instituto Nacional de saúde Dr. Ricardo Jorge)
Doutora Ana Luísa Carvalho, Ph.D. (Universidade de Coimbra)

Setembro, 2013

The research work presented in this dissertation was conducted at:

mRNA Metabolism Group

Departamento de Genética Humana

Instituto Nacional de Saúde Dr. Ricardo Jorge

Av. Padre Cruz

1649-016 Lisboa

Portugal

Gene Expression and Bioinformatics Unit

Center for Biodiversity, Functional & Integrative Genomics

Universidade de Lisboa

Faculdade de Ciências

Campus da FCUL, C2.2.12

Campo Grande

1749-016 Lisboa

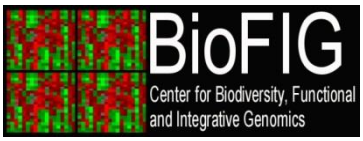
Portugal

Financial support:

Fundação para a Ciência e a Tecnologia

(fellowship SFRH/BD/33462/2008)

Fundação Merck Sharp & Dohme



AGRADECIMENTOS

Foi com enorme privilégio que desenvolvi os trabalhos constantes desta tese e é com elevada satisfação que atinjo esta recta final. Esta tese seria uma página em branco se não tivesse tido a ajuda das várias pessoas que me rodearam ao longo destes anos. Desta forma, é com grande carinho que agradeço a todos os que estiveram ao meu lado e deram o seu apoio precioso para esta fase bastante feliz da minha vida.

Começo por deixar um agradecimento muito especial à minha orientadora Dra. Luísa Romão, por me ter dado a possibilidade de realizar os trabalhos constantes nesta tese no seu laboratório. A sua orientação, disponibilidade, sentido prático e palavras encorajadoras foram absolutamente fundamentais. Agradeço igualmente ao Dr. Sukalyan Chaterjee pela orientação não só científica como também pessoal, pela frontalidade, honestidade e amizade. Deixo aqui igualmente um agradecimento muito especial ao meu grande amigo Bruno, pela sua sensatez, paciência, boa disposição, presença e apoio incondicionais, pelos momentos de lazer partilhados e, acima de tudo, pela amizade. Quero também agradecer à Rafaela, que foi uma confidente e me animou nos momentos mais difíceis. Agradeço-lhe igualmente pelas correcções desta dissertação, pela ajuda no trabalho experimental, pela compreensão, optimismo, boa disposição e amizade. Agradeço também ao Alexandre, por me ter apoiado no trabalho experimental, pela troca de ideias, pela boa disposição, compreensão, companheirismo e, acima de tudo, por ser do Norte. Deixo igualmente um grande agradecimento ao Marco, nosso colaborador, pela partilha de ideias, disponibilidade, boa disposição, espontaneidade e pelos bons momentos passados no laboratório. Agradeço à Ana Morgado, à Filipa Tomé, à Filipa Reis, à Andreia, ao Miguel, ao David, à Cláudia, à Catarina, ao Daniel e à Cristina, por tornarem os dias no instituto muito mais agradáveis e prazerosos. Agradeço também aos colegas do laboratório da

Oncobiologia pelo companheirismo e pelo apoio prestado. Um muito obrigada aos meus amigos Angela, Beto, Marco, Magda, Rafaela, Freitas, Nelson, Rui, Carolina, Diana e Cristiana por perceberem as minhas ausência, pelos momentos de lazer, pelas palavras encorajadoras e, acima de tudo, por me aturarem há tanto tempo. Agradeço também à Lilian, por me ter apoiado na fase da escrita da tese. Deixo aqui igualmente um agradecimento à minha querida irmã, pelas palavras de encorajamento, pelo carinho e por me ter dado dois sobrinhos lindos.

Agradeço ao Filipe pelo seu apoio e presença incondicionais, pelo sentido prático, e acima de tudo, pelo seu amor. Por último agradeço ao meu Pai e à minha Mãe, por serem os meus pilares, por me apoiarem em todas as minhas decisões, por saberem sempre o que é melhor para mim e por acreditarem em mim, muito mais do que eu.

ACKOWLEDGEMENTS

It was a privilege to develop the work presented in this dissertation and is with joy that I reach this final step. This thesis would be a white page without the precious help of several people, to whom I thank with affection.

I would like to specially acknowledge my supervisor, Dra Luísa Romão, for giving me the opportunity to develop my PhD work in her lab, for the guidance, practical sense and kind words in the most difficult moments. I am also thankful to Dr. Sukalyan Chaterjee for the scientific and personal guidance, frontality, honesty and friendship. I dedicate a special word for my dear friend Bruno, for the prudence, patience, sense of humor, unconditional support, joyfull moments and, above all, for the friendship. I am also thankful to Rafaela, for listening and supporting me in the most difficult moments, for the corrections of this thesis and the experimental support, for the understanding, optimism, sense of humor and for the friendship. I want to thank to Alexandre for the scientific support, sense of humor, understanding, friendships and, above all, for being from the North. I would also like to thank to Marco, our collaborator, for the brainstormings, availability, sense of humor, spontaneity and the good moments spent at the lab. I also want to acknowledge to Ana Morgado, Filipa Tomé, Filipa Reis, Andreia, Miguel, David, Cláudia, Catarina, Daniel and Cristina, for turning the working days into pleasure moments. I am also thankful to the Oncobiology lab members for the companionship and support. I want to dedicate a special acknowledgement to my dear friends Angela, Beto, Marco, Magda, Rafaela, Freitas, Nelson, Rui, Carolina, Diana and Cristiana for understanding my absence, for the leisure moments, the supportive words and, above all, for being my friends after all these years. I want to thank also Lilian for being a partner and confident during the thesis writing process. I want to

acknowledge my dear sister, for the supportive words, the affection and for giving me the best nephews.

I want to dedicate a special word to Filipe for the unconditional support, practical sense and more than everything else, for his love. Finally I want to thank my pillars, my mam and dad, for supporting me in all my decisions, for always knowing what is the best for me and for believing in me, much more than I do.

Esta tese é dedicada ao meu pai e à minha mãe.

TABLE OF CONTENTS

ABSTRACT

Regulation of mRNA translation plays a major role in controlling gene expression, since it allows rapid cellular responses to external stimuli without the involvement of transcription, mRNA processing or transport. Translational control can be transcript-specific via regulatory cis-acting elements, such as internal ribosome etry sites (IRESs). mRNA translation initiation driven by IRES elements is independent of some canonical initiation factors that are inhibited by cellular stresses or in some physiological conditions and pathophysiologic settings. Accordingly, IRES-dependent translation allows continued protein synthesis in conditions with repression of the canonical mechanism of mRNA translation and has been reported in several transcripts encoding stress-responsive proteins, oncogenes and tumor suppressor genes. In this work, it is demonstrated that the mammalian (or mechanistic) target of rapamycin (MTOR) and the Δ160P53 protein isoform are expressed through IRES-driven translation.

MTOR is a conserved serine/threonine kinase that integrates signals from growth factor stimulation as well as from cellular nutrient- and energy-status, acting namely on the protein synthesis machinery. Major advances are emerging regarding the effects and regulators of MTOR signaling pathway, however, regulation of *MTOR* gene expression, namely at the translational level, is not well known. Here, it is shown that the 5' untranslated region (5'UTR) of the human *MTOR* mRNA contains an IRES element that allows cap-independent translation of *MTOR*. In addition, it is demonstrated that IRES-dependent translation of *MTOR* is stimulated by hypoxia with associated EIF2α phosphorylation, in a manner that is independent of hypoxia-inducible factor 1α (HIF1α) induction *per se*. The anti- and pro-apoptotic outcomes of the unfolded protein response induced by endoplasmic reticulum (ER) stress also stimulates *MTOR* IRES activity, with a more pronounced effect in the pro-apoptotic

phase with associated EIF2α phosphorylation. Furthermore, it is illustrated that *MTOR* IRES activity is potentiated by MTOR complex 1 (MTORC1) inactivation, suggesting a feedback loop in order to maintain MTOR expression. These data point out a novel regulatory mechanism of *MTOR* gene expression that integrates the protein profile rearrangement triggered by global translational inhibitory conditions. Furthermore, these results give a possible explanation how MTOR signalling is not lost in cellular stress conditions with impaired mRNA translation.

The P53 protein has a fundamental role at restraining tumor development. Upon an insult, P53 develops a protective program that, depending on the stress and/or damage severity, relies either on a pro-survival response including temporary cell cycle arrest and damage repair or it induces cellular senescence, apoptotic or autophagic cell death. The tumor protein P53 (*TP53*) gene orchestrates the formation of several protein isoforms through the use of distinct promoters, alternative splicing and IRES-mediated translation. The P53 protein isoforms act either through modulation of the activity of P53 protein or display cellular functions that are executed in a P53-independent manner. P53 is one of the most studied proteins due to its role as a tumor suppressor and as *TP53* represents one of the most common mutated genes in cancer. Nevertheless, new roles for the P53 family are still arising, with particular emphasis to its protein isoforms. Recently, a new P53 isoform originated by mRNA translation initiation at codon 160, the Δ160P53 protein isoform, was discovered. Nevertheless, the mechanism responsible for its expression was not addressed, as well as its function in the cell. Here, it is demonstrated that expression of the Δ160P53 protein isoform is induced upon cellular overconfluency, and by ER stress through augmented translation. It is identified an IRES element downstream of 160 start codon that is governing cap-independent production of Δ160P53 protein isoform. Interestingly, it is demonstrated

that the 5´UTR of Δ160P53 inhibits the activity of the IRES element that assists its expression. In addition, it is shown that increased phosphorylation levels of EIF2α stimulate the activity of the IRES for Δ160P53. In line with this work, a collaborator group found that the Δ160P53 protein inhibits apoptosis, promotes cell growth and induces malignant transformation.

This study presents the IRESs for MTOR and Δ160P53 as potential new therapeutic targets for treatment of innumerous diseases, such as cancer, associated with hyper-activated MTOR signaling or with augmented expression of Δ160P53, respectively.

Keywords: translation regulation; translation initiation; internal ribosome entry site (IRES); mammalian target of rapamycin (MTOR); Δ160P53.

RESUMO

A regulação ao nível da iniciação da tradução de mRNAs é fundamental no processo de controlo de expressão génica uma vez que permite uma resposta celular rápida face a estímulos externos. Este controlo pode ocorrer de forma específica de transcrito, através de elementos reguladores em cis, tais como *internal ribosome entry sites* (IRESs), que medeiam a tradução de forma independente de alguns factores de iniciação canónicos que são inibidos em condições de stress celular, ou em algumas condições fisiológicas ou patológicas. Desta forma, a tradução dependente de IRES é refractária a condições que inibem a síntese proteica global. Estes elementos encontram-se em transcritos que codificam proteínas responsivas a stress, oncogenes ou supressores de tumor. O trabalho apresentado nesta dissertação mostra que os transcritos que codificam o *mammalian (or mechanistic) target of rapamycin* (MTOR) e a isoforma proteica de P53, Δ160P53, possuem elementos IRESs a regular a sua expressão.

O MTOR é uma serina/treonina quinase conservada que integra sinais provenientes da estimulação por factores de crescimento, assim como dos estados nutricional e energético da célula actuando, nomeadamente, na maquinaria de tradução. Apesar da crescente compreensão acerca dos mecanismos de regulação e efeitos da via de sinalização do MTOR, o controlo da sua própria expressão, nomeadamente ao nível da tradução, permanece largamente desconhecido. Os resultados descritos nesta tese demonstram que a região 5´ transcrita e não traduzida (5'UTR) do mRNA *MTOR* humano contém um elemento IRES que permite a sua tradução de forma independente da estrutura cap. Adicionalmente, demonstra-se que a tradução de *MTOR* mediada por IRES é estimulada em hipoxia com associado aumento da fosforilação de EIF2α e que esta estimulação é independente da indução de *hypoxia-inducible factor* 1α (HIF1α) *per se*. A fase anti-apoptótica da *unfolded protein response* induzida por stress do retículo

endoplasmático (RE) estimula a tradução de *MTOR* mediada por IRES, contudo um efeito mais pronunciado é observado na fase pró-apoptótica com associado aumento da fosforilação de EIF2α. Mostra-se ainda que a inactivação de MTORC1 é acompanhada por estimulação do IRES do *MTOR*, sugerindo um circuito de auto-regulação com o intuito de manter os níveis proteicos de MTOR constantes. Estes resultados demonstram um novo mecanismo regulador da expressão génica de *MTOR*, que integra o rearranjo de perfil proteico observado em condições que inibem globalmente a tradução. Para além disso, os resultados aqui apresentados podem explicar o facto da via de sinalização do MTOR não ser perdida em condições que inibem a síntese proteica.

A proteína P53 possui papel fundamental no impedimento de desenvolvimento tumoral. Em condições de stress, P53 desenvolve um programa protector que, dependendo da severidade do stress e/ou dano causado, poderá promover a sobrevivência celular através da indução de uma paragem temporária do ciclo celular e da reparação dos danos ou promover a inviabilização da célula através da indução de senescência celular ou morte por apoptose ou autofagia. O gene *tumor protein p53* (*TP53*) expressa várias isoformas proteicas através da utilização de diferentes promotores, splicing alternativo ou tradução mediada por IRES, que actuam tanto através da modulação da actividade da proteína P53 como de forma independente desta. A sua função primordial na supressão da tumorigénese e o facto de *TP53* ser um dos genes mais frequentemente mutados em cancro, faz com que este seja um dos genes mais estudados. Contudo, tem-se vindo a verificar que as funções da família de P53 ainda não são totalmente conhecidas e a descoberta de novos membros tem vindo a adensar a complexidade desta família. Recentemente foi descoberta uma nova isoforma proteica originada apartir de iniciação da tradução no codão 160, tendo sido

designada por Δ160P53. Porém, o mecanismo responsável pela sua expressão assim como a sua função permaneceram um mistério. O trabalho explanado nesta dissertação mostra que a expressão de Δ160P53 é induzida por sobre-confluência celular e em stress do RE através de taxas de tradução aumentadas. Adicionalmente, é aqui identificado um elemento IRES a jusante do codão de iniciação 160, o qual é responsável pela expressão da isoforma proteica Δ160P53. Curiosamente demonstra-se que a 5´UTR de Δ160P53 inibe a actividade deste elemento IRES. Para além disso, mostra-se que o aumento da fosforilação de EIF2α estimula a síntese de Δ160P53 mediada por IRES. Na sequência deste trabalho, um grupo colaborador mostrou que a proteína Δ160P53 inibe a apoptose, promove crescimento celular e induz transformação maligna.

O trabalho descrito nesta tese apresenta os IRESs que assistem a síntese de MTOR e Δ160P53 como potenciais novos alvos terapêuticos para o tratamento de várias doenças, tal como cancro, com hiper-activação da via de sinalização do MTOR e expressão aumentada de Δ160P53, respectivamente.

Palavras-chave: regulação da tradução; iniciação da tradução; *internal ribosome entry site* (IRES); mammalian target of rapamycin (MTOR); Δ160P53.

The data presented in this dissertation regarding the MTOR IRES element was submitted to PLOS Genetics. The work regarding the Δ160p53 study was performed in collaboration with Marco Candeias (University of Kyoto, Japan) and a manuscript is under preparation for submission to Nature.

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AFAP	Attenuated familial adenomatous polyposis
AMPK	Adenosine monophosphate-activated protein kinase
APAF1	Apoptotic peptidase activating factor 1
APC	Adenomatous polyposis coli
ATF	Activating transcription factor
ATP	Adenosine diphosphate
BAG1	BCL2-associated athanogen
BAX	BCL2-associated X protein (BAX)
BCL2	B-cell CLL/lymphoma2
BCL2L1	BCL2-like protein 1
BH3	BCL2 homology 3
Bp	Base pairs
CAT1	High affinity cationic amino acid transporter 1
CBP	Cap-binding protein
CDK	Cyclin-dependent kinase
CDKN1	Cyclin-dependent kinase inhibitor 1
cDNA	Complementary DNA
CHOP	CCAAT/enhancer-binding protein homologous protein
CHX	Cyclohexamide
CIRP	Cold inducible RNA binding protein
CRC	Colorectal cancer
C-terminal	Carboxi-terminal
DAP5	Death-associated protein 5
DBD	DNA binding domain
DDR	DNA damage response
DENR	Density-regulated protein
DEPTOR	DEP domain containing MTOR-interacting protein
DKC1	Dyskeratosis congenita 1
DMSO	Dimethyl sulfoxide
EEF2	Eukaryotic translation elongation factor 2
EGFP	Enhanced Green Fluorescent Protein
EIF	Eukaryotic translation initiation factor
EJC	Exon-junction complex
EMCV	Encephalomyocarditis virus
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
FAP	Familial adenomatous polyposis
FGF2	Fibroblast growth factor 2
FKB12	FK-506 binding protein of 12 kDa
FLuc	Firefly luciferase
FRB	FKBP12-rapamycin binding
GADD34	Growth arrest and DNA damage-inducible protein-34
GAIT	Interferon γ-activated inhibitor of translation
GCN2	General control non-derepressible-2
GDP	Guanosine diphosphate
GSK3β	Glycogen synthase kinase 3β

GTP	Guanosine triphosphate
h	Hours
HBB	Human β-globin
HCV	Hepatitis C Virus
HIF	Hypoxia-Inducible Factor
HnRNP	Heterogeneous nuclear ribonucleoprotein
HRI	Heme-regulated inhibitor
Ig	Immunoglobulin
IGR	Intergenic region
IRE	Iron response element
IRE1	Inositiol-requiring protein 1
IRES	Internal ribosome entry sites
IRP	Iron-regulatory protein
IRS1	Insulin receptor substrate 1
ITAF	IRES trans-acting factor
JNK	C-Jun N-terminal kinase
LAMB1	Laminin beta 1
M	Molecular weight marker
MCT1	Monocarboxylate transporter 1
Met-tRNAi	Initiator methionyl-tRNA
miR	MicroRNA
MLH1	MutL homolog 1
MLST8	Mammalian lethal with sec-13 protein 8
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein particle
MSIN1	Mammalian stress-activated map kinase-interacting protein 1
MTOR	Mammalian or mechanistic target of rapamycin
MTORC	MTOR complex
MYC	V-myc avian myelocytomatosis viral oncogene homolog
MYCL	V-myc avian myelocytomatosis viral oncogene lung carcinoma derived homolog
NES	Nuclear export signal
NLS	Nuclear localization signal
Nt	Nucleotide
N-terminal	Amino-terminal
O-C	Over-confluency
OD	Oligomerization domain
OIS	Oncogene-induced senescence
ORF	Open reading frames
P	Peptidyl
PABP	Poly(A)-binding protein
PAGE	Polyacrilamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PAS	Predicted splice Acceptor Site
P-bodies	Processing bodies
PCBP1	Poly(rC) binding protein 1
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDCD	Programmed cell death protein

PDGF	Platelet-derived growth factor
PDS	Predicted splice Donor Site
PERK	PKR-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3-kinase
PIC	Pre-initiation complex
PKB	Protein kinase B
PKR	Protein kinase RNA-dependent
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein
Pol	Polymerase
PRAS40	Proline-rich Akt substrate 40 kDa
PRTE	Pyrimidine-rich translational elements
PSF/SFPQ	PTB associated splicing Factor
PTB	Polypyrimidine tract-binding protein
PV	Poliovirus
PVDF	Polyvinylidene difluoride
P53RE	P53 response elements
RAG	Ras-related GTP-binding protein
Rap	Rapamycin
RAPTOR	Regulatory-associated protein of mammalian target of rapamycin
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of MTOR
RLU	Relative light units
RLuc	Renilla luciferase
RP	Ribosomal protein
rRNA	Ribosomal RNA
RSK	Ribosomal S6 kinase
RT	Reverse transcriptase
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
S6K	S6 kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Ser	Serine
SGK1	Serum- and glucocorticoid- induced protein Kinase 1
SKAR	S6K1 Aly/REF-like target
SL	Stem loop
SNAT2	Sodium-coupled neutral amino acid transporter
TAD	Transactivation domains
TC	Ternary complex
TEL2	Telomere maintenance 2
TG	Thapsigargin
Thr	Threonine
TMEM132A	Transmembrane protein 132A
TNFα	Tumour necrosis factor α
TOP	5´terminal oligopyrimidine tracts
TP53	Tumor protein p53
tRNA	Transfer RNA
TSC	Tuberous Sclerosis Complex
TTI1	TELO2 interacting protein 1

TU	Tunicamycin
U	Upstream
UPR	Unfolded protein response
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
Vh	Vehicle
XBP1	X-box binding protein 1
X-DC	X-linked dyskeratosis congenita
XIAP	X-linked inhibitor of apoptosis
YB1	Y-box binding protein 1
β-gal	β-galactosidase
4EBP	Eukaryotic translation initiation factor 4E binding protein
4ET	Eukaryotic translation initiation factor 4E transporter

I. INTRODUCTION

III.1. Eukaryotic mRNA translation initiation

Eukaryotic gene expression involves a myriad of tightly regulated events. The fact that steady-state transcript levels only partially correlate with protein abundances in several organisms (de Sousa Abreu et al., 2009) evokes the possibility that post-transcriptional regulatory mechanisms strongly affect gene expression. Regulation at the level of mRNA translation, a process that leads to protein synthesis from genomic information, offers a rapid response to external stimuli without the involvement of transcription, mRNA processing or transport. mRNA translation is a multistep pathway encompassing initiation, elongation, termination and ribosome recycling steps. All phases of mRNA translation are subjected to regulatory mechanisms, although an overwhelming bias towards regulation at the initiation step is observed (reviewed in Sonenberg and Hinnebusch, 2009)

This thesis will focus on mRNA translation initiation and its regulatory mechanisms.

mRNA translation initiation requires several factors, including the ribosome which is composed of a small subunit (40S) and a large subunit (60S) that together form the 80S ribosome. The ribosome is the primary site of protein synthesis and recognizes and binds the cap structure, a 7-methyl guanosine that is linked to the 5´ terminal nucleoside of the mRNA through an inverted 5´-5´triphosphate bridge (Shuman, 2001). This binding occurs through one of the various eukaryotic translation initiation factors (EIFs) that are keys players in this process. Attached to the ribosome is one specific transfer RNA (tRNA), the initiator methionyl-tRNA (Met-tRNAi) (Cooper, 2000) that binds to the appropriate codon, AUG in the majority of cases, by complementary base pairing.

The goal of translation initiation is to form an elongation-competent 80S ribosome in which the anticodon of the Met-tRNAi is base-paired with the initiation codon of the mRNA located in the ribosomal peptidyl (P) site. The mechanism underlying this process for the majority of transcripts comprises formation and recruitment of the pre-initiation complex (PIC) to the 5´ cap structure of an activated mRNA, followed by ribosomal scanning of the 5´UTR in the 5´ to 3´direction, that proceeds until a start codon in a favorable context is found. The commitment of scanning-arrested PICs to the initiation codon is mediated by EIFs displacement which is followed by 60S ribosomal subunit joining resulting, ultimately, in 80S initiation complex formation (reviewed in Aitken, 2012) (Figure I.1).

Met-tRNAi is delivered to the 40S ribosomal subunit by the EIF2 to which it binds. Further binding of GTP to EIF2 leads to the formation of the ternary complex (TC) (Levin et al., 1973). During ribosome recycling EIF1, EIF1A and EIF3 attach the 40S ribosomal subunit and, in conjunction with EIF5, induce a ribosomal "open conformation" to accommodate the TCs (Asano et al., 2001; Kolupaeva et al., 2005; Majumdar et al., 2003). Attachment of the TC to the 40S ribosomal subunit, bound to EIF1, EIF1A, EIF3 and EIF5, generates the PIC. The EIF4F complex is a key player in the subsequent loading of PIC into the mRNA. It is composed of the cap-binding protein EIF4E, the ATP-dependent RNA helicase EIF4A and the scaffoldding protein EIF4G. EIF4E interacts with the 5´cap structure and, along with EIF4G, recruits EIF4A to the mRNA to unwind secondary structures located within the 5´UTR in a ATP-dependent manner, preparing a landing site for PIC (reviewed in Aitken, 2012).

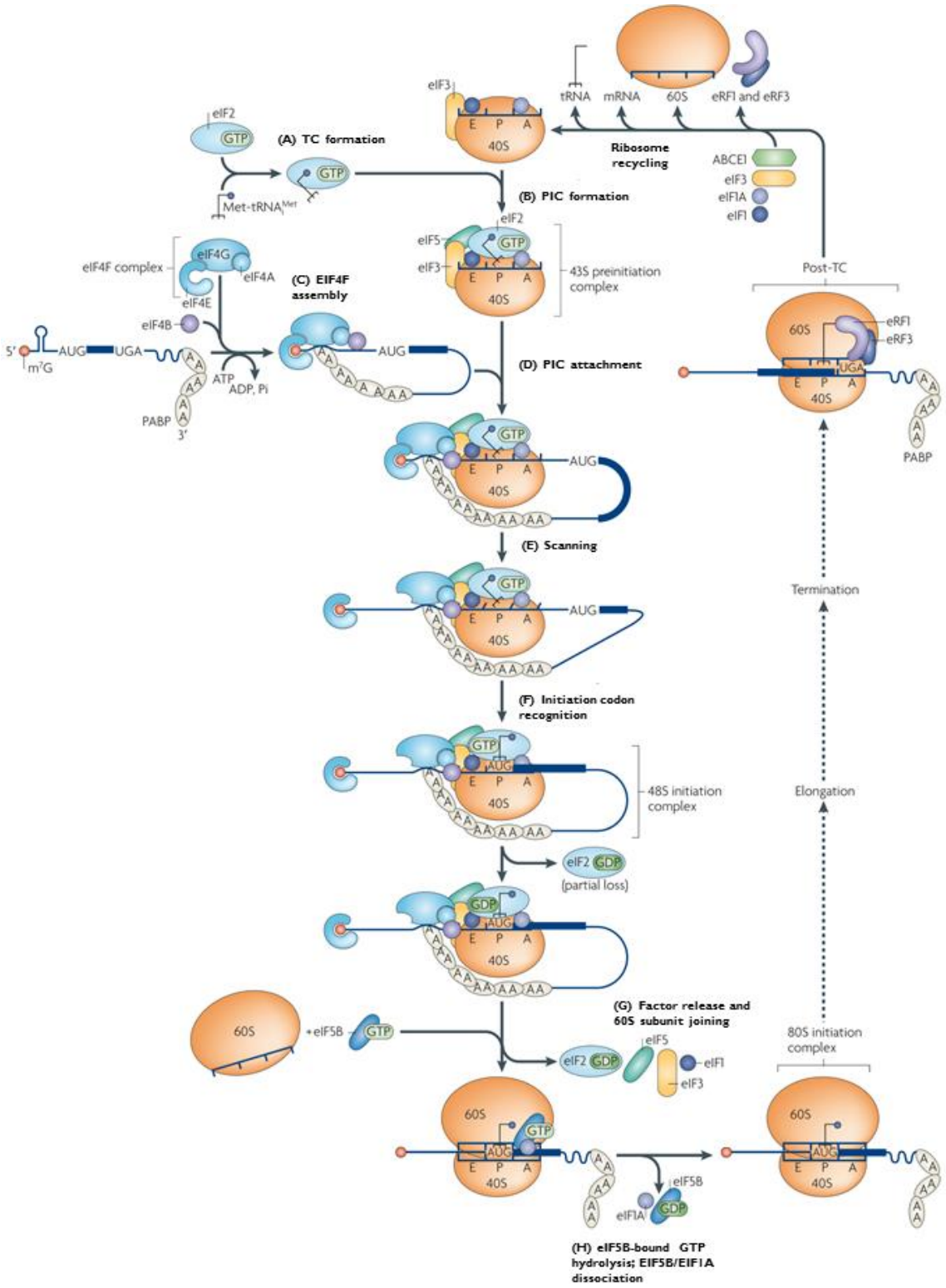


Figure I.1. Canonical eukaryotic translation initiation. Steps of canonical eukaryotic translation initiation. Translation initiation occurs after recycling of post-termination complexes (post-TCs), which occur through release of associated factors and ribosomal subunit dissociation. **(A)** The ternary complex (TC), composed by initiator methionyl-tRNA (Met-tRNAi) and a GTP-bound EIF2, is formed and **(B)** is recruited to the 40S ribosomal subunit bound to EIF1, EIF1A, EIF3 and EIF5, leading to formation of the pre-initiation complex (PIC). **(C)** Meanwhile the EIF4F complex,

composed by EIF4E, EIF4G and EIF4A, is assembled in the mRNA 5´terminus, through EIF4E-5´cap structure interaction. EIF4G binds with the polyA-binding protein (PABP) generating a closed-loop mRNA configuration. EIF4F and the auxiliary protein EIF4B unwinds the 5´region of the circularized transcript for subsequent **(D)** PIC attachment, which occurs through interaction between EIF3 and EIF4G/EIF4B. **(E)** The assembled PIC scans the 5´untranslated region (5´UTR) through secondary structure unwinding and ribosomal movement in the 5´to 3´direction, until **(F)** an initiation codon in a favorable context is found, with which the Met-tRNAi anti-codon base pairs, switching the scanning PIC to a closed conformation with EIF1 release. EIF5 triggers EIF2-bound GTP hydrolysis and EIF2-GDP partial release. **(G)** EIF5B triggers displacement of the remaining EIF2-GDP, EIF1, EIF4B, EIF4F, EIF5 and EIF3 and 60S ribosomal subunit joining, leading to 80S complex formation. **(H)** Subsequent hydrolyzes of EIF5B-bound GTP leads to its dissociation and the following release of EIF1A allows 80S complex to start the elongation step. *Adapted with permission from Jackson et al., 2010.*

The RNA helicase activity of EIF4A is stimulated by EIF4B and EIF4H that increases the affinity of EIF4A for ATP and the mRNA and possibly by stabilization of the 5´UTR region where the ribosome initially binds and by disabling secondary structure´s refolding (Abramson et al., 1988; Lindqvist et al., 2008; Marintchev et al., 2009; Richter et al., 1999; Rogers et al., 1999, 2001). In addition, binding to EIF4G also stimulates EIF4A helicase activity by modulation of the affinity of this initiation factor for ATP and by induction of productive conformational changes (Marintchev et al., 2009; Schütz et al., 2008). It is deemed that eI4G is also involved in the interaction between EIF4E and the 5´ terminal cap, since when this initiation factor is coupled to EIF4E, the affinity between the latter and the cap structure is increased (Gross et al., 2003; Volpon et al., 2006). By interacting with poly(A)-binding protein (PABP), a protein that binds to the polyadenylated 3´end of the transcript and the cap-associated EIF4E, EIF4G brings the 5´ and 3´ends of the mRNA together, originating a closed-loop configuration (Wells et al., 1998).

It has been suggested that EIF4F attachment to the mRNA relies on multiple interactions rather than on absolute contributions of each initiation factor. The cap structure is deemed to inspect the success of this network of interactions (Mitchell et

al., 2010). The redundancy of such interactions is demonstrated by the fact that EIF4G seems to be capable of interacting directly with mRNA in an EIF4E-independent manner, suggesting EIF4F assembly in the mRNA might also occurs solely via EIF4G (Kaye et al., 2009; Mitchell et al., 2010; Park et al., 2011; Yanagiya et al., 2008). On the other hand, EIF4G depletion leads to reduction rather than abolishment of translation initiation and it has been proposed that EIF4G function to enhance the EIF4F-mRNA interaction, instead (Hinton et al., 2007; Park et al., 2011; Ramírez-Valle et al., 2008). Furthermore, the absolute role of PABP in translation initiation has been questioned, since it seems to be most important under competitive conditions (Svitkin et al., 2009). The PIC component EIF3 serves as a ribosome-EIF4F bridge, by binding particularly to EIF4G and allowing PIC assembly onto the transcript (Hinnebusch, 2006; LeFebvre et al., 2006). EIF4B is also able to interact with EIF3 and a role for this initiation factor in the recruitment of PIC has also been demonstrated (Dmitriev et al., 2003; Méthot et al., 1996; Mitchell et al., 2010).

Once at the 5´ terminal of the mRNA, PIC scans the 5´UTR by unwinding the secondary structures and moving in the 5´ to 3´direction, presumably through a base-by-base process. Ribosomal backward movement has also been observed, although over a course of very few nucleotides (Kozak, 1991; Matsuda and Dreher, 2006). The process of mRNA unwinding requires EIF4A and is ATP-dependent, as previously mentioned, whereas ribosomal movement *per se* is independent of factors involved in mRNA unwinding , but is dependent on EIF1 and EIF1A (Passmore et al., 2007; Pestova and Kolupaeva, 2002). Ribosomal scanning and codon inspection proceeds until the correct initiation site is recognized, a process that is determined by start codon nucleotide context and its position relative to 5´ terminal of the mRNA. An optimal context is GCC(A/G)CCAUGG (Kozak context), in which the positions -3 and +4 (A

of AUG is set as +1) are crucial, specially a purine at position -3 (Kozak, 1984, 1986a, 1987a). Usually the scanning PIC recognizes the first AUG in a good Kozak context (Kozak, 1991). The fidelity of this process is assured by EIF1 that prevents recognition of non-AUG codons, AUGs in poor contexts or located in the first eight 5´ terminal nucleotides. Furthermore, EIF1 induces PIC dissociation in case of incorrect start codon selection (Pestova and Kolupaeva, 2002; Pestova et al., 1998). Upon pairing of the mRNA start codon with Met-tRNAi anticodon, now localized in the P site of the ribosome, and EIF1 dissociation, PIC acquires a close conformation (Maag et al., 2005), a process that is deemed to involve interactions between the nucleotides at -3 and +4 positions interact with PIC components (Pisarev et al., 2006).

Subsequent commitment to a start codon occurs when EIF5, an EIF2-specific GTPase-activating protein, elicits hydrolysis of EIF2-bound GTP which decreases EIF2 – Met-tRNAi affinity and, thus, triggers partial EIF2-GDP dissociation (reviewed in Aitken, 2012). Dissociation of the remaining EIF2-GDP and EIF1, along with 60S ribosomal subunit joining, is mediated by the GTPase EIF5B (Pestova et al., 2000; Unbehaun et al., 2004). However it is thought that EIF3 is also released at this stage in an EIF5B-dependent manner, it has been suggested that this initiation factor might remain attached to 80S-elongating complexes, according to its role in reinitiation events (Pöyry et al., 2007). Formation of 80S initiation complex prompts hydrolysis of EIF5B-bound GTP leading to its dissociation and subsequent release of EIF1A (Acker et al., 2009).

III.2. Translational regulation at the initiation level

Translation regulation encompasses both non-specific mechanisms affecting the overall pool of transcripts, and mRNA-targeted controls through specific mRNA elements.

III.2.1. Overall mRNA translation initiation regulation

The formation and/or regeneration of TCs and the assembly of functional EIF4F complexes are two important points of control of mRNA translation initiation.

I.2.1.1. Control of ternary complex formation

One major target of translation initiation regulation is the TC formation and regeneration. Following each round of translation, the dissociated EIF2 needs to be recharged with GTP, as the GDP-bound EIF2 form is unable to bind Met-tRNA. This exchange is performed by the guanine exchange factor EIF2B. When the α subunit of EIF2 is phosphorylated, the affinity between EIF2α and EIF2B increases, leading to sequestering of EIF2B from the free pool. Since EIF2B is less abundant than EIF2, subsequent rounds of translation will be inhibited by TC formation impairment (reviewed in Pavitt, 2005). Phosphorylation of EIF2α occurs on the conserved Ser51 and is triggered by several stresses such as hypoxia, amino acid starvation, viral infection, heat shock and ER stress, by differential activation of four mammalian kinases: heme-regulated inhibitor (HRI), protein kinase RNA-dependent (PKR), PKR-like endoplasmic reticulum kinase (PERK) and general control non-derepressible-2 (GCN2) (reviewed in Wek et al., 2006). HRI is activated by iron or heme deficiency, heat, osmotic and oxidative stresses in erythroid cells (reviewed in Chen and Perrine, 2013); as long as the interferon-inducible PKR is induced by binding to double-stranded RNAs and is important for the anti-viral response (Zheng and Bevilacqua, 2004); PERK becomes activated by the unfolded protein response (UPR) triggered by ER stress (Harding et al., 1999) whereas induction of GCN2 is triggered by amino acid starvation (reviewed in Dever and Hinnebusch, 2005).

Although the outcome of conditions inducing EIF2α phosphorylation is overall protein synthesis reduction, translation initiation of some transcripts is refractory or even augmented by this phenomenon, allowing a gene expression reprogramming towards translation of transcripts encoding proteins involved in stress response and translational recovery (reviewed in Proud, 2005).

I.2.1.2. Control of EIF4F formation

As previously mentioned, the 40S ribosomal recruitment to mRNAs is dependent on EIF4E-cap binding. Simultaneously, EIF4E binds to EIF4G generating, in combination with EIF4A, the EIF4F complex. Assembly of this complex into the mRNA is another major target of regulation of mRNA translation initiation (Duncan et al. 1987). This regulation is namely performed by the EIF4E-binding proteins (4EBPs), the EIF4E transporter (4ET), EIF4E-homologous protein (4EHP), phosphorylation events of EIF4G and the programmed cell death protein 4 (PDCD4).

The competitive fitness of 4EBPs binding to EIF4E determines whether EIF4E binds to EIF4G or remains sequestered by them, since both initiation factors share the EIF4E-binding site (Haghighat et al., 1995; Mader et al., 1995; Marcotrigiano et al., 1999). Hypo-phosphorylated 4EBPs display high binding affinity to EIF4E and thus disables EIF4F formation with concomitant translation initiation inhibition (Lin et al., 1994; Pause et al., 1994). The phosphorylation status of 4EBPs is governed by several kinases, such as MTOR (Beretta et al., 1996; Brunn et al., 1997; Burnett et al., 1998; Hara et al., 1997), P110α and P110γ (Foukas and Shepherd, 2004) and is regulated in response to growth factors, nutrient, oxygen and energy status. 4EBPs activity is involved in cell cycle control and proliferation, since depletion of 4EBPs selectively inhibits synthesis of proliferation-promoting proteins and transcripts encoding proteins involved in cell

cycle progression (Dowling et al., 2010). In Drosophila, 4EBPs regulate tissue aging by removal of damaged and aggregated proteins that accumulate during muscle aging possibly through the autophagy/lysosome system (Demontis and Perrimon, 2010). Translation of transcripts harboring IRES elements is usually refractory to 4EBP phosphorylation (Thoreen et al., 2012).

EIF4F complex formation is further impaired by 4E-T, that binds to EIF4E and sequesters it in the nucleus and P-bodies (a place of mRNA degradation or storage) (Dostie et al., 2000; Ferraiuolo et al., 2005). In addition, the EIF4E homolog protein (4EHP), that is unable to form EIF4F complexes, inhibits EIF4E attachment into the cap structure by competitive binding to the latter (Tee et al., 2004).

Additionally, EIF4G phosphorylation results in decreased association of this initiation factor with EIF4E (Ling et al., 2005; Pyronnet et al., 2001). For instance, the p21 protein (Cdc42/Rac)-activated kinase 2 binds and phosphorylates EIF4G, leading to a decrease in the interaction between EIF4G and EIF4E in addition to decreased EIF4E-cap association (Ling et al., 2005). Furthermore, caspase and viral cleavage of EIF4G also impairs its ability to bind EIF4E, thus inhibiting EIF4F complex formation (Haghighat et al., 1996; Marissen and Lloyd, 1998).

EIF4A is also subjected to control, namely by PDCD4 that binds to this initiation factor, blocking its interaction with the scaffold protein EIF4G and its RNA helicase activity (Chang et al., 2009; Suzuki et al., 2008; Yang et al., 2003).

III.2.2. Translation regulation mRNA-specific

Cis-acting elements endow transcripts with differential sensitivity to alterations in the activity of translational machinery components allowing mRNA-specific control. Those elements include, namely, secondary and tertiary structures, iron response elements,

interferon γ-activated inhibitor of translation elements, upstream open reading frames and internal ribosome entry sites.

III.2.2.1. Secondary and tertiary RNA structure

Hairpins and pseudoknots within transcripts 5´UTRs display an intrinsic ability to affect translation. These structures inhibit mRNA translation initiation with an efficiency that is determined by its position relative to the cap structure and its thermodynamic stability: when in close proximity, a hairpin with a Gibbs free energy of -30kcal/mol inhibits mRNA translation initiation very efficiently, whereas a hairpin located further away, needs to be more stable (less than -50kcal/mol) to affect the translational apparatus (Kozak, 1986b). In addition, the GC content of a hairpin influences *per se* its ability to affect mRNA translation (Babendure et al., 2006). Those structures are particularly present in mRNAs encoding oncogenes, transcription factors, growth factors and their receptors.

III.2.2.2. Iron response elements

Iron response elements (IREs) are small elements found in the 5´ or 3´UTRs of several transcripts encoding proteins involved in iron metabolism. These elements are 28-nucleotides long and are responsible for regulation of mRNA translation or stability in response to iron levels (reviewed in Wallander et al., 2006). IREs located at the 5´ UTRs regulate mRNA translation initiation whereas those found in the 3´UTR control mRNA turnover (Theil, 1993). Iron deficiency triggers binding of the iron-regulatory proteins (IRPs) to the IREs elements which block PIC assembly thus leading to translation inhibition. On the other hand, when iron is abundant, IRPs are subjected to

posttranslational modifications that either diminish the affinity for the IREs elements or promote their own degradation by the proteasome (Guo et al., 1995).

III.2.2.3. Interferon γ-activated inhibitor of translation element

The interferon γ-activated inhibitor of translation (GAIT) element is a bipartite stem–loop structure located at the 3′UTR of several transcripts involved in the inflammatory response, to which the GAIT complex binds, inhibiting protein translation (reviewed in Mukhopadhyay et al., 2009). This complex is composed by the ribosomal protein (RP) L13a (RPL13a), the glutamyl-prolyl tRNA synthetase, NS1-associated protein 1, and glyceraldehyde 3-phosphate dehydrogenase proteins (Mazumder et al., 2003; Sampath et al., 2004). The effect of GAIT complex on mRNA translation occurs through inhibition of PIC recruitment. The GAIT complex binds to the GAIT element and to the EIF3-binding site of EIF4G, thus inhibiting the EIF4G-EIF3 interaction (Arif et al., 2009; Kapasi et al., 2007). This process occurs in a PABP and poly(A) tail-dependent manner (Kapasi et al., 2007; Mazumder et al., 2001).

III.2.2.4. Upstream open reading frames (uORFs) and translation reinitiation

The presence of uORFs within transcripts 5′UTRs has been reported in several mammalian genes, some of which with a negative impact in the expression of the main ORFs-encoded protein (Calvo et al., 2009). Translation initiation at uORF AUG is governed by the Kozak consensus sequence criteria (Kozak, 1984, 1986a, 1987a) and generally leads to reduction of main ORF expression (Calvo et al., 2009). A poor uORF initiation codon context triggers the leaky scanning phenomenon, in which the ribosome either recognizes the AUG and initiates translation or misses it and

continues scanning until a favorable context is found, determining a small or even absent effect of uORF at main ORF expression. On the other hand, a good AUG context might lead to uORF translation initiation with relative good efficiency. After translation of an uORF, some ribosomes remain attached to the mRNA, resume scanning and reinitiate translation at a downstream start codon (Kozak, 1987b). In the majority of cases, only the 40S ribosomal subunit remains attached into the mRNA and resumes scanning after translation of an uORF. Neverthless, a recent work suggests the involvement of 80S ribosomes in some reinitiation events (Skabkin et al., 2013). The efficiency of translation reinitiation at a downstream ORF is determined by several aspects namely the uORF length and complexity, distance between uORF stop codon and the downstream initiation codon, TCs availability, uORF stop codon surrounding sequence and ribosome stalling by the uORF-encoded peptide.

It has been demonstrated that reinitiation only occurs if EIF4F complex and EIF4B participate in the upstream translation event, or at least EIF4G p50 fragment, EIF4A and EIF4B (Poyry et al., 2004). Furthermore, in yeast, EIF3 remains bound to 80S during translation elongation and this is critical for increased translation reinitiation of post-terminating 40S ribosomes (Szamecz et al., 2008). Accordingly, downstream reinitiation occurs when the uORF is permissive for maintenance of ribosome-associated initiation factors, which is determined by the time spent by the ribosome to translate it. Downstream reinitiation after translation of a short and unstructured uORF might be highly efficient in opposition to a long or structured uORF (Kozak, 2001). This is demonstrated by the fact that a decrease in reinitiation efficiency was observed upon expansion of uORF length from 13 to 33 codons (Kozak, 2001) and a completely abolishment of translation reinitiation was observed when this uORF was lengthened to 55 codons (Luukkonen et al., 1995). Furthermore, insertion of a stable

RNA hairpin also decreases reinitiation rate by translation elongation pausing (Kozak, 2001). A particular case of reinitiation after a long uORF was reported at a viral mRNA. In this case, a cis-acting element consisting of the 87 nucleotides located immediately upstream of the uORF stop codon that is able to bind both 40S ribosomal subunit and EIF3, was mandatory for translation reinitiation at the second ORF, possibly by capturing some of the EIF3-40S complexes generated during ribosome disassembly (Poyry et al., 2007).

Besides the time required for uORF translation, reinitiation efficiency is also dependent on the distance between uORF stop codon and the downstream initiation codon. For each round of translation EIF2 is inactivated, so reinitiation must *de novo* recruit an active GTP-bound EIF2. Nevertheless, scanning resumption starts appreciably before a new TC is acquired as it occurs rapidly after translation termination (Kozak 1987b, 2001). In accordance, the intercistronic space must be long enough to allow TCs recruitment. It has been demonstrated that a 79-nucleotide (nt) distance between uORF stop codon and the downstream AUG allows translation of the second ORF as efficiently as if no uAUG was present (Kozak, 1987b). Additionally, the availability of active TCs, also plays an important role in the reinitiation efficiency. While increased EIF2α phosphorylation reduces translation in an overall level (reviewed in Dever, 2002), the presence of uORFs of appropriate length and position might result in increased protein synthesis instead. Augmented expression of activating transcription factor 4 (ATF4) and ATF5 by increased phosphorylation of EIF2α occurs by such mechanism (Vattem and Wek, 2004; Zhou et al., 2008). The 5´UTRs of both transcripts harbor two uORFs, a 5´ terminal short uORF1 and a downstream longest uORF2 which overlaps with *ATF4* or *ATF5* main ORF, respectively. Under normal conditions, i.e., high ternary complex levels, after translation of uORF1, ribosome

resumes scanning and re-acquires active TCs in time to reinitiate at uORF2. Translation reinitiation at *ATF4* or *ATF5* main ORF is compromised as it would imply backwards scanning over a longer distance that ribosome can handle (Kozak, 2001; Vattem and Wek, 2004; Zhou et al., 2008). When EIF2α phosphorylation increases, after translation of uORF1, ribosomes only acquire TC after bypassing uORF2 and before *ATF4* or *ATF5* main AUG, thus increasing their translation (Vattem and Wek, 2004; Zhou et al., 2008).

Reinitiation efficiency might also be reduced by ribosome stalling during uORF translation elongation or termination. In some cases, this stalling is caused by the uORF encoded peptide itself. It seems that it is not the peptide sequence that determines this effect, as no consensus sequence determining ribosome stalling has been identified until now. Ribosome stalling might interfere with downstream translation reinitiation not only because the stalled ribosome is disabled to reach the downstream AUG, but also because the newly loaded ribosomes find a barrier (reviewed in Morris and Geballe, 2000).

III.2.2.4.1. **Non-AUG initiation codons**

In rare cases, mRNA translation may initiate at non-AUG codons that differ at a single nucleotide from the bona fide initiator (Peabody, 1989). The recognition of a non-AUG codon as a translational initiation site is severely dependent on an optimal Kozak context, requiring both a purine at position -3 and a "G" at position +4 (Kozak, 1991), and generally needs additional signals to be recognized, such as downstream stem-loops (Kozak, 1989a). The influence of the sequence context seems to be not restricted to positions -3 and +4, as some cases of dependency on nucleotides located at other positions had arised (Chen et al., 2008; Kozak, 1989a). Kozak tested

translation initiation at six different non-AUG initiators and found that GUG provided the more efficient translation but it was only functional when preceded by the sequence CCACC (Kozak, 1989a). This suggests that the imperfect codon-anticodon interaction is compensated by contact with nearby nucleotides, in particular with a purine at position -3 and a G in +4 (Kozak, 1991). As it occurs with AUG initiators in a weak Kozak consensus sequence, translation at non-AUG initiation codons is enhanced by downstream stem-loops located at specific positions. A stem-loop located at a distance of 17 and, to a less extent, 11 nucleotides downstream of the coding sequence beginning enhances translation efficiency (Kozak, 1990).

III.2.2.5. Internal ribosome entry site

The scanning model of translation initiation was proposed on 1978 by Marilyn Kozak (Kozak, 1978) and, since then, it prevails as the mechanism used by the vast majority of the transcripts. Though, early observations pointed out that this mechanism fails to explain how certain mRNAs are translated. In 1988, it was demonstrated that some viral uncapped mRNAs are not only efficiently translated, but also are able to compete with host mRNAs for the translational machinery, under viral infection (Jang et al., 1988; Schneider and Shenk, 1987). Furthermore, ribosome profiling of cells infected with poliovirus (PV), that inhibits translation initiation in an EIF4G-cleavage-dependent manner, showed that about 3% of the mRNAs remained associated with polysomes (Johannes et al., 1999) many of them encoding proteins involved in cell stress responses. Particularly, under this translational inhibitory condition, translation of transmembrane protein 132A (*TMEM132A*) (previously kwon as binding protein 1 (*BIP*) transcript was found to be enhanced (Sarnow, 1989). Similar observations were found in other conditions with associated reduction of cap-dependent translation, such as

mitosis (Qin and Sarnow 2004) and apoptosis (Bushell et al., 2006). These data suggested a translational advantage of certain mRNAs, upon unfavorable conditions for the scanning-mediated mRNA translation. Then, two independent studies observed that PV and encephalomyocarditis virus (EMCV) 5´UTRs induce translation of a second cistron expressed by a dicistronic RNA, which lead to the conclusion that those sequences directly bind the 40S ribosomal subunit without the involvement of the mRNA 5´ terminal (Jang et al., 1988; Pelletier and Sonenberg, 1988). Subsequent confirmation of these data came from the observation that circle RNAs containing EMCV 5´UTR efficiently recruit the translational machinery which was able to translate for consecutive rounds (Chen and Sarnow, 1995). First known as "ribosome landing pads", those sequences were after termed IRES and classified into "viral" and "cellular" IRES. Subsequent studies demonstrated that IRES elements are present in other RNA viruses (Locker et al., 2011) as well as DNA viruses (Tahiri-Alaoui et al., 2009). The first cellular IRES discovered was within *TMEM132A* 5´UTR (Macejak and Sarnow, 1991) and after then, several cellular IRESs have been discovered (reviewed in Martínez-Salas et al., 2012).

III.2.2.5.1. **IRES features**

An IRES is a cis-acting element consisting of a highly structured RNA sequence that attracts and binds the 40S ribosomal subunit without requiring the cap-structure. Although IRES elements are usually located within the 5´UTR of a transcript, some IRESs are also (Allam and Ali., 2009; Candeias et al., 2006) or exclusively (Jaag et al., 2003) located in the mRNA coding region, in which they trigger production of truncated proteins. Generally, IRES-containing transcripts have long 5´UTRs (reviewed in Baird et al., 2006) with high GC content, stable secondary structures, uAUGs or

uORFs (reviewed in Martínez-Salas et al., 2012). However, these are not universal characteristics and a great variability of features and mechanisms of action is observed between viral and cellular IRESs as well as among the discovered cellular IRESs. Whereas the viral IRESs are about 300-600 nts long, the cellular counterparts are shorter with an average of 100-200 nts (reviewed in Komar and Hatzoglou, 2005). IRESs of viruses fold in highly ordered RNA secondary and tertiary structures, generating several domains, whose structural integrity is required for IRES activity (eg., Honda et al., 1996). Usually, the different domains display distinct functions in the process of recruiting the translational machinery (Jang and Jan, 2010; Serrano et al., 2009). On the other hand, cellular IRESs fold in a less stable RNA structure (Xia and Holcik, 2009) and their activity seem to rely on the action of individual modules that cooperatively act to attract the translational apparatus, though displaying IRES activity *per se* (eg., Coldwell et al., 2000; Stoneley et al., 1998). Nevertheless, cases of structural similarity of such modules with the ones found in viral IRESs have been reported. IRES elements within the *TMEM132A* and fibroblast growth factor 2 (*FGF2*) transcripts harbor a Y-type stem-loop structure followed by a small hairpin upstream of the initiation codon similar to the one found in the picornavirus IRESs (Le and Maizel, 1997). Furthermore, v-myc avian myelocytomatosis viral oncogene homolog (*MYC*) and v-myc avian myelocytomatosis viral oncogene lung carcinoma derived homolog (*MYCL*) IRESs possess pseudoknots (Jopling et al., 2004; Le Quesne et al., 2001), a tertiary motif important for IRES activity of some viruses (Rijnbrand et al., 1997). On the other hand, a negative influence of RNA secondary and tertiary structures has been proposed for some cellular IRESs. For instance, destabilization of some pseudoknots of *MYC* IRES stimulates its activation (Le Quesne et al., 2001). Furthermore, 40S ribosomal subunit attachment to the IRES element of the apoptotic

protease-activating factor 1 (*APAF1*) mRNA occurs in a single-stranded region (Mitchell et al., 2003). Another study demonstrated that yeast and fruit fly IRESs with weak secondary structures are more active than those containing strong secondary structures (Xia et al., 2009). Those findings show that cellular IRESs do not share with viral IRESs a predictable RNA structure stability-function relation.

The enrichment of IRESs in GC nucleotides is not also an universal finding, as demonstrated by the fact that IRESs elements of seven yeast genes required for invasive growth possess an A-rich stretch immediately upstream of AUG (poorly structured sequence), that is required for IRES activation (Gilbert et al., 2007). This element is a binding site for the poly(A) binding protein (PAB1). In addition, the activity of the unstructured IRESs elements found on the fruit fly transcripts encoding Reaper, Hid, Grim and the Heat shock 70kDa protein is positively correlated with its content on adenine residues (Hernandez et al., 2004; Vazquez-Pianzola et al., 2006).

III.2.2.5.2. **Mechanism of action**

Translation initiation driven by an IRES element relies on cap-independent ribosomal recruitment to the vicinity of the start codon, which is generally assisted by initiation proteins that may include canonical EIFs and/or a battery of proteins called IRES trans-acting factors (ITAFs). The requirement for both EIFs and ITAFs varies significantly between the different IRESs discovered so far. Even viral IRESs display differences in the translational machinery composition (reviewed in Martínez-Salas et al., 2012). Furthermore, IRES-mediated translation initiation might be totally independent of ribosomal scanning and allow direct positioning of AUG at the ribosomal P-site or rely on a "land and scan" mechanism that involves ribosomal scanning, in the 5´-3´ direction, from the landing site to the initiation codon (Belsham and Jackson, 2000).

IRES-dependent translation is, by definition, independent of the 5´ terminal cap structure, so it is not surprising that the majority of IRESs (both cellular and viral) operate in a manner that is independent on the cap-binding protein EIF4E (reviewed in Hellen and Sarnow, 2001). Few exceptions to this were reported for IRESs elements of hepatitis A virus and ring finger protein 1B, whose activation requires the EIF4E-EIF4G interaction (Ali et al., 2001; Boutsma et al., 2008). Indeed, it has been proposed that EIF4E has a negative effect on IRES-dependent translation, since it potentiates the competitive fitness of capped mRNAs for EIF4F complex recruitment. When the EIF4E levels drop, the remaining EIF4G/EIF4A complex, which has lower affinity for capped mRNAs and higher for IRES-containing mRNAs (Lomakin et al., 2000), are preferentially recruited by the IRES element (Svitkin et al., 2005). This model was proposed based on the EMCV IRES behavior, though the growing list of IRES that are stimulated upon conditions with impaired EIF4E (Thoreen et al., 2012), might suggest that this is a common mechanism for IRES dependent translation.

The requirement for other EIFs is more variable and, for viral IRESs, determines a categorization into groups and types, along with its secondary structure and the position of the ribosome landing site relative to the initiation codon. The viral IRESs are divided into the *Picornaviridae*, *Flaviviridae* and *Dicistroviridae* groups and the former is further divided into four different types, type I-IV. IRESs elements of PV and EMCV are the prototypes of type 1 and 2 IRESs, respectively, and require EIF4A and the central domain of EIF4G for recruitment of PIC, which is enhanced by the action of EIF4B (Andreev et al., 2007; Belsham, 1992; de Breyne et al., 2009; López de Quinto et al., 2001). IRESs belonging to type 1 use the "land and scan" mechanism, whereas EMCV IRES-mediated translation relies on the "land and start" mechanism (Belsham and Jackson, 2000; Kaminski et al., 1990, 1994). In Hepatitis C Virus (HCV) IRES-driven

translation, the prototype of the type 3 IRESs, the 48S complex is directly assembled at the initiation codon, in a manner that is dependent on EIF3. In this IRES, the action of EIF2 might be substituted by EIF2D or EIF2A or monocarboxylate transporter 1 (MCT1) in combination with density-regulated protein (DENR) (Dmitriev et al., 2010; Kim et al., 2011; Skabkin et al., 2010). IRESs belonging to type 4, prototyped by the intergenic region (IGR) IRESs, directly interact either with the 40S ribosomal subunit or with 80S ribosomes, in a manner that is independent of EIFs, but is dependent on the IRES structural conformation (Costantino et al., 2008; Wilson et al., 2000a). Among the IGR IRES pseudoknot domains that interact with the ribosomal subunits, the one occupying the P-site mimics the Met-tRNAi anticodon stem loop and a start codon (Costantino et al., 2008).

The involvement of EIFs in IRES-mediated translation of cellular mRNAs is more random. For instance, *TMEM132A* IRES requires the EIF4G C-terminal that binds EIF4A (Thoma et al., 2004), *MYC* and v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (*MYCN*) IRESs are dependent on EIF4G C-terminal, EIF4A and EIF3, whereas *MYCL* IRES requires the EIF4F complex, PABP and EIF3 (Spriggs et al., 2009). The aforementioned A-rich family of yeast IRESs requires EIF4G and is strongly dependent on PABP (Gilbert et al., 2007). Additionally, some IRESs, such as those within the v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (*SRC*), X-linked inhibitor of apoptosis (*XIAP*), high affinity cationic amino acid transporter 1 (*CAT1*), *TMEM132A*, pim-1 oncogene (*PIM1*), platelet-derived growth factor-2 (*PDGF2*), vascular endothelial growth factor (*VEGF*) and *MYC* transcripts might operate in an EIF2-independent manner, as its activity is not inhibited by increased phosphorylation levels of EIF2α (Allam and Ali, 2009; Fernandez et al., 2002; Gerlitz et al., 2002; Thakor and Holcik, 2011). In accordance with its independence on some

EIFs, IRES-driven translation circumvents the inhibitory effect of physiological, stress or pathophysiological conditions that usually inhibit cap-dependent translation, such as those inhibiting EIF4F complex formation or TC regeneration.

ITAFs are RNA-binding proteins that regulate IRES-driven translation and the vast majority is also involved in other biological processes such as transcription, RNA transport, splicing and stability (Pacheco et al., 2008; Vazquez-Pianzola et al., 2005; Weinlich et al., 2009). ITAFs might be positive or negative regulators of IRES-dependent translation and their mechanism of action is deemed to rely on folding of the IRES element in an RNA configuration that is either beneficial or disadvantageous for translational apparatus binding. Additionally, ITAFs may also assist canonical EIFs in ribosomal recruitment or even display this role in an EIF-independent manner (reviewed in Komar and Hatzoglou, 2011). This diversity of roles of ITAFs in modulating IRES activity is exemplified by the stimulatory effect of Upstream of N-ras (UNR) binding to *APAF1* IRES, which induces a structural rearrangement of this element that triggers binding of polypyrimidine tract-binding protein (PTB) (Mitchell et al., 2003), allowing ribosome to land in an unfolded RNA segment. On the other hand, the ITAF RNA-binding motif protein 4 (RBM4) stimulates IRES activity by inciting EIF4A recruitment to IRES-containing transcripts, such as of the *MYC* and B-cell CLL/lymphoma2 (*BCL2*) mRNAs (Lin et al., 2007). Most ITAFs, such as the one of the heterogeneous nuclear ribonucleoprotein (HnRNP) group, are able to shuttle between the nucleus, where they normally reside, and the cytoplasm, as they harbor nuclear export sequences (Michael et al., 1997; reviewed in Piñol-Roma, 1997). It has been demonstrated that ITAFs subcellular localization plays a role in modulating IRES activity. For instance, *MYC*, *APAF1*, *VEGF*, *FGF2* and *XIAP* IRESs are affected by HnRNP A1 cellular cytoplasmic relocalization (Cammas et al., 2007). Similarly, chemotoxic

stress, a condition that induces translation initiation inhibition, is accompanied by maintenance of BCL2-associated athanogen (*BAG1*) IRES activity and concomitant cytoplasmic redistribution of BAG1 ITAFs PTB and poly(rC) binding protein 1 (PCBP1) (Dobbyn et al., 2008). Furthermore, the cytoplasmatic localization of RNA-binding protein 4 is essential for its ITAF activity (Lin et al., 2007). This ITAF subcellular relocalization might have opposite outcomes on modulation of different IRESs as demonstrated by the positive and negative effects displayed by cytoplasmic accumulation of hnRNP A1 on IRES activity of *FGF2* and *XIAP* transcripts, respectively (reviewed in Lewis and Holcik, 2007). The influence of ITAF subcellular redistribution on IRES-dependent translation might imply that nuclear located ITAFs either bind transcripts harboring IRESs elements, thus inhibiting access to the cytoplasm-located translational machinery, or remain unassociated from IRES-containing mRNAs allowing them to migrate to the cytoplasmic compartment where they can be translated (Semler and Waterman, 2008). In both models, an appropriate signal would trigger cytoplasmic relocalization of nuclear located ITAFs (reviewed in Lewis and Holcik, 2007). Posttranslational modifications of ITAFs might also influence its subcellular localization and, concomitantly, its effect on IRES-driven translation. This is demonstrated by the phosphorylation-triggered subcellular redistribution of HnRNP A1 (Van Oordt et al., 2000). Among the several discovered ITAFs, PTB has been proposed to be an "universal ITAF" as it regulates various IRESs namely PV, EMCV, *APAF1*, *BAG1*, cyclin-dependent kinase inhibitor 1B (*CDKN1B*) (Cho et al., 2005; Grover et al., 2008; Jang and Wimmer, 1990; Mitchell et al., 2005; Pestova et al., 1991; Pickering et al., 2003, 2004).

The ribosome itself might also influence IRES-mediated translation, as demonstrated by the fact that the ribosomal RNA (rRNA) pseudouridylation status affects binding of

IRESs to ribosomes leading to alterations of IRES-dependent translation (Jack et al., 2011). Furthermore, it has been shown that rRNA methylation compromises translation initiation via cellular but not viral IRESs, through inhibition of 80S initiation complex formation (Basu et al., 2011).

Additionally, a role for 3´UTR in assisting IRES-mediated translation has been reported (Dobrikova et al., 2003; López de Quinto et al., 2002), suggesting that mRNA circularization might also play a role in IRES-dependent translation.

III.2.2.5.3. **Biological significance of IRESs**

Viruses have strategies to be able to synthesize their own proteins, considering they lack a translational apparatus. Providing that viral IRES-containing transcripts are not translated by the cap-dependent mechanism, one strategy relies on inhibition of host cap-dependent translation and use of IRES-mediated translation, allowing a competitive advantage for the translational machinery resulting in prevalence of viral translation (reviewed in Schneider and Mohr, 2003). For instance, picornavirus infection results in cleavage of host EIF4G and PABP, resulting in selective repression of host cap-dependent translation in opposition to viral IRES-mediated translation that remains active (Rodríguez Pulido et al., 2007). On the other hand, most of their cellular counterparts use both mechanisms of translation initiation (Johannes and Sarnow, 1998; Pinkstaff et al., 2001).

Some stress, physiological and pathophysiological conditions are associated with reduced cap-mediated translation and increased IRES-driven translation (Graber and Holcik, 2007; Silvera et al., 2009; Spriggs et al., 2008). Those conditions include nutrient limitation, temperature shock, DNA damage response, hypoxia, ER stress, as well as apoptosis, mitosis, hibernation and tumorigenesis.

Starvation, even of a single essential amino acid, is accompanied by protein synthesis reduction (Everson et al., 1989). Nevertheless, transcript and protein levels of sodium-coupled neutral amino acid transporter (SNAT2) are elevated through the actions of augmented EIF2α phosphorylation and IRES-activation, respectively (Gaccioli et al., 2006). Additionally, the same condition prompts stabilization and augmented translation of the *CAT1* transcript. mRNA is stabilized by binding of the cytoplasmic redistributed HuR protein to an AU-rich element (ARE) within *CAT1* 3´UTR, as long as the increment of CAT1 protein synthesis is achieved by translation initiation through an uORF-activated IRES element, in a EIF2α-dependent manner (Yaman et al., 2005). Both SNAT2 and CAT1 proteins are components of the system transport A, a neutral amino acid transport system, which is stimulated by amino acid depletion (reviewed in McGivan and Pastor-Anglada, 1994).

The cold inducible RNA binding protein (CIRP) is involved in the cellular response to cold shock, a condition that is characterized by overall decrease of protein synthesis (reviewed in Sonna et al., 2002). In cold shock conditions (32°C), *CIRP* translation is increased by an IRES element within a transcript with the full-length 5´UTR that is produced upon those conditions (Al-Fageeh and Smales, 2009).

The DNA damage response (DDR) encompasses a series of events to promote damage repair or trigger cell death according to the nature or severity of the insult (Jackson and Bartek, 2009). Similarly to the aforementioned cellular stresses, DNA damage is accompanied by reduction of the cap-dependent translation rates (Powley et al., 2009), although, some transcripts with important roles in the DDR continue to be translated, as it occurs with the serine hydroxymethyltransferase 1 (SHMT1). This protein is a member of the thymidylate biosynthetic pathway that is involved in DNA repair (Anderson and Stover, 2009) and it is produced upon UVC exposure through

an IRES element (Fox and Stover, 2009). In addition, the transcripts encoding P53 and BCL2 are IRES-driven translated upon cellular treatment with the DNA damage-inducing agent etoposide (Sherrill et al., 2004; Yang et al., 2006).

Caspase-mediated cleavage of EIF4G, EIF4B and 4EBP1 (Bushell et al., 1999, 2001; Tee and Proud, 2002) and alterations in the phosphorylation levels of EIF2α and 4EBP1 culminate in protein synthesis inhibition during apoptotic conditions (reviewed in Clemens et al., 2000). A role for IRES-driven translation in the cell fate decision upon adverse conditions has been proposed, as transcripts encoding both proteins with anti- and pro-apoptotic functions have IRES elements (Holcik et al., 2000a). Transcripts whose translation is kept under apoptotic stimulus include those encoding MYC (Stoneley et al., 2000a), APAF1 (Coldwell et al., 2000), XIAP (Holcik et al., 1999). Deregulated expression of MYC triggers apoptosis (reviewed in Hoffman and Liebermann, 1998) and it has been demonstrated that maintenance of MYC protein levels under tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is achieved by IRES-driven translation (Stoneley et al, 1998, 2000a). The IRESs of the *APAF1* and *XIAP* transcripts, encoding a pro- and an anti-apoptotic protein, respectively, are activated by different apoptotic stimulus. *APAF1* IRES is stimulated by etoposide- induced apoptosis (Nevins et al., 2002) and it has been suggested that the IRES element within the *APAF1* transcript accounts for the protein levels maintenance upon those conditions (Coldwell et al., 2000). In turn, IRES activity of *XIAP* is increased upon serum starvation, low-dose γ-irradiation and treatment with interleukin-6 (Holcik et al., 1999, 2000b; Yamagiwa et al., 2004) but not in etoposide or thapsigargin-induced apoptosis (Nevins et al., 2002; Warnakulasuriyarachchi et al., 2004). The apoptotic-stimulus specificity of IRES activation of those transcripts might determine whether the cells commit suicide or resume growth.

The G2/M transition is characterized by a substantial decrease in overall protein synthesis, namely through inhibition of the EIF4F assembly, or EIF2α phosphorylation (Bonneau and Sonenberg, 1987; Datta et al., 1999; Pyronnet et al., 2001) and an increase in IRES-mediated translation (Ramirez-Valle et al., 2010). It has been shown that the transcript of the ornithine decarboxylase (ODC), a key enzyme in the synthesis of polyamines, harbors an IRES element which is active during G2/M but not in the G1/S phase (Pyronnet et al., 2000). In addition, a p58-kDa isoform of the cyclin-dependent kinase 11 (CDK11), is produced through an IRES element located within *CDK11* mRNA coding region, during G2/M (Cornelis et al., 2000). Impairment of IRES-driven synthesis of $CDK11^{p58}$ leads to impaired cytokinesis, aneuploidy and tumorigenesis (Wilker et al., 2007). The increase of IRES-dependent translation in mitosis has been attributed to phosphorylation of the MTORC1-component RAPTOR (Ramírez-Valle et al., 2010). Furthermore, a role for 14-3-3σ has also been described. During mitosis, 14-3-3σ binds to several EIFs, namely EIF4B and EIF2α and the elongation factor EF1α; in its absence, the switch towards IRES-dependent translation rather than cap-dependent translation is not observed (Wilker et al., 2007).

Mammalian hibernation is characterized by impairment of overall translation (van Breukelen and Martin, 2001). Translation initiation mediated by IRESs elements is deemed to play a role in this adaptive process as transcripts harboring IRES elements are preferentially loaded into polysomes during arousal of hibernating ground squirrels in comparison to non IRES-containing mRNAs (Pan and van Breukelen, 2011).

In summary, IRES-mediated translation is the preferred mechanism of translation of some transcripts during physiological or stress conditions which are inhibitory to the canonical mechanism. Accordingly, IRES-driven translation integrates translational

reconfiguration programs that are triggered to allow cells to bypass the adverse condition or commit irreversibly-damaged cells to death.

III.2.2.5.4. Importance of IRES-mediated translation in cancer

Deregulation of mRNA translational control has been associated with tumor development. Cellular transformation is often accompanied by a translational reconfiguration characterized by augmented overall protein synthesis, which results in increased cell growth and division, in combination with translational modulation of specific transcripts encoding proteins with oncogenic properties (reviewed in Silvera et al., 2010). This is accomplished namely by abnormal expression or activity of canonical initiation factors, tRNAs or translational regulatory proteins, as well as an increase in rates of ribosomal biogenesis and ribosomal subunits amounts (reviewed in Ruggero, 2012; Silvera et al., 2010). In particular, overexpression, increased activity and phosphorylation of EIF4E has been associated with malignant transformation. The role of EIF4E in human cancer development is attributed to its role both on overall protein synthesis and on selective translation of transcripts encoding proteins involved in cell cycle progression, angiogenesis, cell growth, proliferation and apoptosis (Mamane et al., 2007; Topisirovic et al., 2003; Wendel et al., 2007). This selectivity of translation relies namely on the competitive advantage for EIF4E binding that mRNAs with highly structured 5´UTRs gain when this factor is in excess (Koromilas et al., 1992a). Several transcripts encoding proto-oncogenes, growth factors and growth-regulated proteins have long and structured 5´UTRs (reviewed in Willis, 1999). Inhibition of EIF4E, by elevated levels or hypophosphorylation of 4EBP1, are associated with good survival outcome of patients with ovarian and breast cancers and childhood rhabdomyosarcoma (Armengol et al., 2007). Induction of apoptosis is observed upon

ectopic expression of a 4EBP1 phosphorylation site-mutant into breast carcinoma cells (Avdulov et al. 2004). A role for EIF2α in tumorigenesis has been suggested by the fact that deregulated TC formation triggers cell transformation (Koromilas et al. 1992b). However, the influence of EIF2α phosphorylation status on tumorigenesis seems to be context- and cancer stage-dependent. In some studies, inhibition of EIF2α phosphorylation triggers malignant transformation (Donzé et al., 1995; Koromilas et al., 1992b), while in other conditions, mice with inactivated PERK or phosphorylated EIF2α develop smaller and slowing growth tumors that display higher levels of apoptosis in hypoxic areas (Bi et al. 2005). Nevertheless, in other settings, inactivation of PKR did not influence tumorigenesis (Abraham et al., 1999; Yang et al., 1995).

The balance between cap- and IRES-mediated translation displays a role in tumorigenesis. The initial burst in protein synthesis observed upon cellular transformation occurs mainly through the cap-dependent mechanism (Bellodi et al., 2010). On the other hand, upon oncogene-induced senescence (OIS), a decrease in overall cap-dependent translation and increase in mRNA translation via IRESs elements is observed. One of the transcripts whose IRES-mediated translation is favored during OIS is *TP53* (Bellodi et al., 2010a). In cells with mutations in the dyskeratosis congenita 1 (*DKC1*) gene, a signature of the X-linked dyskeratosis congenita (X-DC) condition, this preferential IRES-dependent synthesis of P53 is impaired allowing cells to bypass the OIS-induced cell cycle arrest (Bellodi et al., 2010a). Furthermore in this condition, the activity of the IRESs elements within the transcripts encoding the cell cycle inhibitor CDKN1B and of the BCL2-like protein 1 (BCL2L1) and XIAP is also impaired (Yoon et al., 2006). Deregulation of IRES-mediated synthesis of the tumor suppressors CDKN1B and P53 account for the increased cancer susceptibility of X-DC patients (Bellodi et al. 2010a,b; Montanaro et al. 2010; Yoon et al., 2006). It is interesting that

although the DKC1-encoded dyskerin is a pseudouridine synthase that alters rRNA, in X-DC only IRES but not cap-dependent translation is impaired (Yoon et al., 2006).

Deregulation of EIFs expression or activity contributes to the balance between cap and IRES-mediated translation, as it occurs in large advanced breast cancers, in which overexpression of 4EBP1 and EIF4G assists a hypoxia-mediated switch towards IRES-driven translation at the expense of cap-dependent translation. The transcritps selectively translated via IRESs elements encode proangiogenic factors, proteins involved in hypoxia response and cell survival, such as HIF1α, VEGFA and BCL2. This translation reconfiguration is required for tumor angiogenesis and progression (Braunstein et al., 2007). Overexpression of EIF4G is also observed in inflammatory breast cancer, in which it triggers IRES-mediated translation namely of *VEGFA* and catenin (cadherin-associated protein), delta 1 (*CTNND1*). VEGFA expression accounts for the high levels of angiogenesis and resistance to hypoxia observed in this tumor type, while CTNND1 protein maintenance is responsible for preservation of tumor cell emboli, a set of clusters of cancer cells linked by catenin delta-1-regulated E-cadherin (Silvera et al., 2009). The transcription factor MYC is commonly hyperactivated in human cancer types (Gardner et al., 2002) and it has been demonstrated that the oncogenic activity of MYC is intimately related to its role in protein synthesis, through distinct effects on cap- versus IRES–dependent translation. Whilst hyperactivation of this transcription factor augments overall protein synthesis rates, it impairs activity of some IRES elements (reviewed in Ruggero, 2009). Furthermore, the translation of *MYC* transcript itself is commanded by an IRES element (Stoneley et al., 1998), whose activity is enhanced by a C-T mutation in the DNA region of this cis-regulatory element. This increase in IRES-driven translation is responsible for MYC overexpression in multiple myeloma cells, in which it is

associated with enhanced cell proliferation (Chappell et al., 2000). The mutant *MYC* IRES element is a stronger mediator of mRNA translation initiation due to its higher affinity to the ITAFs PTB and the overexpressed Y-box binding protein 1 (YB1) (Cobbold et al., 2010).

Aberrant expression or subcellular localization of ITAFs might also account for the role of IRES in tumorigenesis. For instance, PTB overexpression has been observed in some neoplasias, such as endometrial adenocarcinoma tumors (Wang et al., 2008). Loss of epithelial cell polarity and acquisition of mesenchymal features occurs at late stages of tumor progression, by a phenomenon referred as epithelial-mesenchymal transition (EMT), by which cells gain migratory and invasive properties (Thiery et al., 2009). It has been demonstrated that YB1 induces EMT of breast epithelial cells by activation of IRES-dependent translation of the EMT-inducer snail family zinc finger 1 (*SNAI1*). Other transcription factors involved in regulation of epithelial, growth-related and mesenchymal genes are induced by YB1 (Evdokimova et al., 2009). Furthermore, Laminin beta 1 (LAMB1), a protein involved in the cell-extracellular matrix interaction (Patarroyo et al., 2002), is also synthesized through an IRES element. Augmented protein synthesis of LAMB1 during EMT correlates with enhanced *LAMB1* IRES activity (Petz et al., 2007), through a PDGF-dependent increase in the cytoplasmatic redistribution of Lupus La protein during this phenomenom (Petz et al., 2011, 2012). These data might indicate that this augmented IRES-mediated translation of *LAMB1* has a role in tumor cell migration and metastization.

III.3. Mammalian or mechanistic target of rapamycin

A natural compound with antifungal and antiproliferative activities was isolated from a strain of *Spreptomyces hygroscopicus* and was called rapamycin after the place of its

discovery, Rapa Nui (Easter Island) (Vézina et al., 1975). Later, the gene encoding the protein responsible for the functions of this macrolide was found in yeast, and was named target of rapamycin (*TOR*) (Heitman et al., 1991). Shortly after, its mammalian ortholog (MTOR) was discovery and cloned (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995) and since then, intensive research has been made uncovering the overwhelming functions of this protein, revealing that MTOR is fundamental for proper cellular homeostasis. MTOR is a serine/threonine protein kinase of the phosphatidylinositol 3-kinase (PI3K)-related kinase family that integrates signals from growth factor stimulation and hormone receptor activation, as well as from cellular nutrient- and energy-status, regulating cell growth and metabolism (reviewed in Hay and Sonenberg, 2004).

III.3.1. MTOR complexes

MTOR is the core of two complexes, MTOR complex 1 (MTORC1) and MTORC2 (Figure I.2) which display different functions according to distinct downstream targets (reviewed in Hay and Sonenberg, 2004). In conjunction with the regulatory-associated protein of MTOR (RAPTOR), the mammalian lethal with sec-13 protein 8 (MLST8), the DEP domain containing MTOR-interacting protein (DEPTOR), the TELO2 interacting protein 1 (TTI1)/telomere maintenance 2 (TEL2) complex and the prolin-rich PKB substrate 40 kDa (PRAS40), it forms MTORC1 (Hara et al., 2002; Jacinto et al., 2004; Kaizuka et al., 2010; Kim et al., 2002, 2003; Peterson et al., 2009; Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007; Wang et al., 2007).

A

B

Components					
Common to both complexes		MTOR	Catalytic subunit		
		MLST8	Unknown function; required for MTORC2 activity		
		DEPTOR	Inhibitor		
		TTI1/TEL2 complex	Complex assembly and stability		
Complex specific		MTORC1		MTORC2	
		RAPTOR	Substrate recruitment; complex localization and assembly	RICTOR	Substrate recruitment and complex assembly
		PRAS40	Inhibitor	MSIN1	Complex assembly; interaction with SGK1
				PROTOR 1/2	Complex function towars SGK1

Figure I.2. MTOR complexes. (A) Schematic representation of MTOR complex 1 (MTORC1) and MTORC2. **(B)** Functions of shared and specific components of the MTOR complexes. The serine/threonine protein kinase mammalian or mechanistic target of rapamycin (MTOR) is the catalytic subunit of both complexes. The mammalian lethal with sec-13 protein 8 (MLST8) is a component of both complexes and is essential for MTORC2 activity, in opposition to MTORC1. The DEP domain containing MTOR-interacting protein (DEPTOR) inhibits MTORC1 and MTORC2 activity. The TELO2 interacting protein 1 (TTI1) and its interacting partner telomere maintenance 2 (TEL2) are involved in MTORC1 and MTORC2 complex assembly and stability. Specific of MTORC1 is the regulatory-associated protein of mammalian target of rapamycin (RAPTOR) which regulates MTORC1 assembly, localization and substrate recruitment; and the proline-rich PKB substrate 40 kDa (PRAS40) that inhibits MTORC1 activity. MTORC2 possess the rapamycin-insensitive companion of MTOR (RICTOR) that regulates substrate binding and complex assembly; the mitogen-activated protein kinase 2-associated protein 1 (MSIN1) that is also involved in complex assembly and is important, in conjunction with protein observed with RICTOR 1 and 2 (PROTOR1/2), for MTORC2 function towards its target serum- and glucocorticoid- induced protein kinase 1 (SGK1).

MTORC2 is composed by MLST8, DEPTOR, TTI1/TEL2 complex, the rapamycin-insensitive companion of MTOR (RICTOR), the mitogen-activated protein kinase 2-associated protein 1 (MSIN1) and protein observed with RICTOR 1 and 2 (PROTOR1/2) (Frias et al., 2006; Jacinto et al., 2004, 2006; Kaizuka et al., 2010; Kim et

al., 2003; Pearce et al., 2007, 2011; Peterson et al., 2009; Sarbassov et al., 2004; Thedieck et al., 2007) (Figure I.2).

III.3.1.1. MTOR complex 1

MTORC1 is responsive to amino acid availability, growth factor stimulation, cellular stress and energy levels and, upon a stimulatory signal, it promotes cell growth, proliferation and survival by increasing mRNA translation, lipid biosynthesis, hepatic ketogenesis, mitochondria and ribosome biogenesis, and by reducing autophagy and lysosomal biogenesis (reviewed in Laplante and Sabatini, 2013). A representation of the upstream regulators of MTORC1 and MTORC2 is displayed in Figure I.3.

A key regulator of MTORC1 is the Ras homolog enriched in brain (RHEB) that, when bound to GTP, binds and activates MTORC1 kinase activity (Inoki et al., 2003; Long et al., 2005). RHEB is localized in the endomembrane system and the specific point of interaction and activation of MTORC1 is in the lysosomal surface (Sancak et al., 2010).

Regarding the other positive signals, MTORC1 activation is dependent on the presence of amino acids, specially leucine and arginine (Blommaart et al., 1995; Hara et al., 1998). Amino acids accumulated in the lysosomal lumen signals to MTORC1 through the vacuolar H^+ -ATPase that interacts, in the lysosomal surface, with the Ragulator complex (Bar-Peled et al., 2012; Sancak et al., 2010; Zoncu et al., 2011).

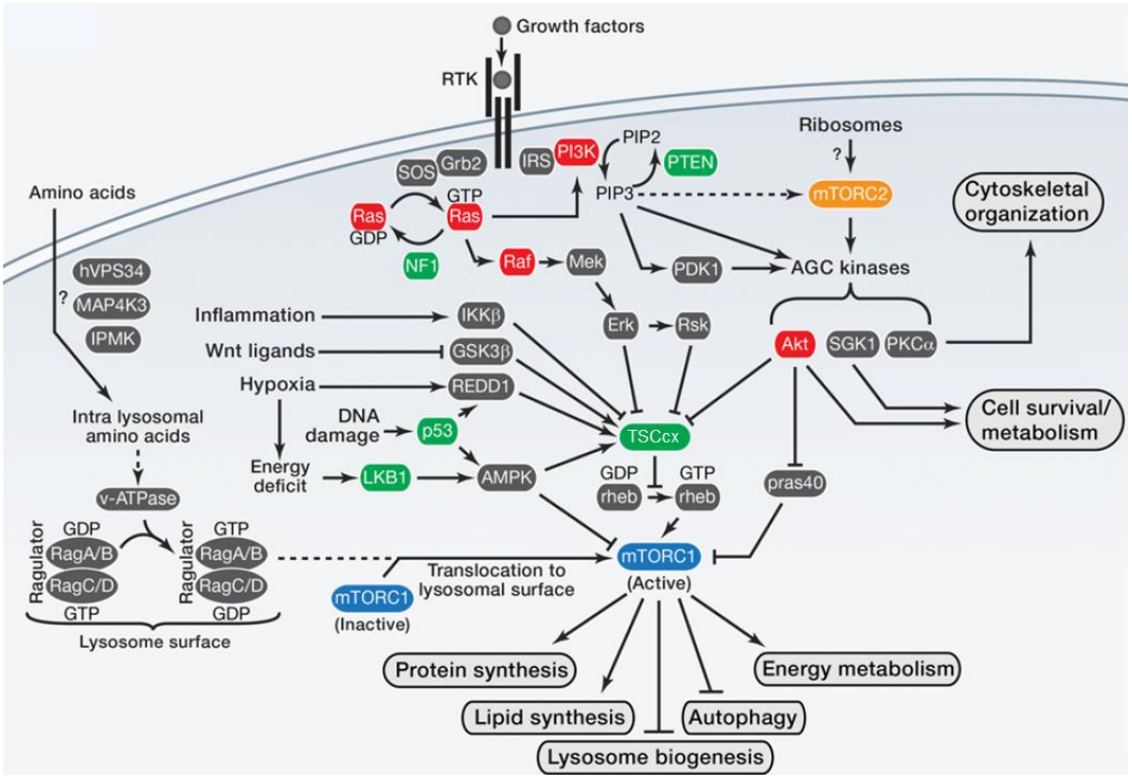


Figure I.3. MTOR signaling. MTOR complex 1 (MTORC1) is regulated by growth factors, cytokines, the canonical Wnt pathway, cellular stress, energy and oxygen levels that signals to MTORC1 mainly through regulation of the tuberous sclerosis complex (TSC) complex (TSCcx), that inhibits the MTORC1-activator Ras homolog enriched in brain (RHEB). Amino acids directly activate MTORC1 by inducing its colocalization with RHEB. Activated MTORC1 induces protein and lipid synthesis and energy metabolism whilst inhibits lysosome biogenesis and autophagy. MTORC2 is regulated by growth factor stimulation which results in increased interaction of this complex with ribosomes. Upon activation, MTORC2 positively regulates actin cytoskeleton organization, cell survival and metabolism. *Adapted with permission from Laplante and Sabatini, 2012.*

In turn, the Ragulator complex interacts with Ras-related GTP-binding protein (RAG) GTPases, and the guanine nucleotide exchange factor activity of this complex induces the loading of the RAG GTPase RAG B or RAG A with GTP. This GTP loading stimulates the binding of GTP-bound RAG B-containing heterodimers with RAPTOR (Bar-Peled et al., 2012; Sancak et al., 2008, 2010). These series of events lead to MTORC1 activation by physical relocation of MTORC1 to the cellular compartment in which RHEB resides, in a way that the binding of RAG GTPases with Ragulator relocates them to the lysosomal surface and binding of MTORC1 to GTP-bound RAG

B or RAG A relocates MTORC1 from the cytoplasm to this subcellular compartment (where RHEB is located) (Sancak et al., 2010).

The stimulatory role of RHEB towards MTORC1 is inhibited by the Tuberous Sclerosis Complex (TSC), composed of the harmatin or tuberous sclerosis 1 (TSC1), tuberin or TSC2 and Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7 (TBC1D7) (Dibble et al., 2012; van Slegtenhorst et al., 1998). This complex binds to RHEB and stimulates its GTPase activity, thus converting it into its inactive GDP-form (Castro et al., 2003; Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). Although the amino acid-dependent activation of MTORC1 is independent on TSC complex (Roccio et al., 2006; Smith et al., 2005), the majority of the upstream signals regulate MTORC1 activity through this complex.

Growth factors, such as insulin, activate the receptor tyrosine kinases (RTKs) that stimulate the PI3K and RAS pathways leading, ultimately, to TSC inactivation through its phosphorylation by the protein kinase B (PKB), the extracellular-signal-regulated kinase 1/2 (ERK1/2) and the ribosomal S6 kinase (RSK) (Inoki et al., 2002; Ma et al., 2005; Manning et al., 2002; Potter et al., 2002; Roux et al., 2004). The PI3K/PKB signalling pathway also inhibit the PRAS40, reverting its inhibitory effect on MTORC1 activity, in a TSC complex-independent fashion (Sancak et al., 2007; Thedieck et al., 2007; Vander Haar et al., 2007; Wang et al., 2007). In addition, RSK also phosphorylates raptor, thus activating MTORC1 activity (Carriere et al., 2008). A series of feedback loops are created by MTORC1 activation, inhibiting the RTKs signals (Hsu et al., 2011; Tzatsos and Kandror, 2006; Um et al., 2004; Yu et al., 2011). For instance, upon MTORC1 activation, S6K1 phosphorylates the insulin receptor substrate 1 (IRS1), an activator of the PI3K signalling, which results in its degradation (Harrington et al., 2004; Um et al., 2004). In addition, IRS1 is also a node between

MTORC1 and MTORC2 signals, since MTORC2 induces its degradation (Kim et al., 2012).

Low ATP levels are sensed by adenosine monophosphate-activated protein kinase (AMPK) that induces ATP formation and reduces its consumption (Kahn et al., 2005). Activation MTORC1 is inhibited by this kinase (Kimura et al., 2003), through phosphorylation and activation of TSC2 (Inoki et al., 2006). In addition, AMPK directly phosphorylates RAPTOR which induces the binding of 14-3-3 to the MTOR binding partner, leading to MTORC1 inactivation (Gwinn et al., 2008).

Some cytokines, such as the tumour necrosis factor α (TNFα), also regulates MTORC1 through phosphorylation of TSC1 and inhibition of the TSC complex, by the inhibitor of nuclear factor K-B kinase-β (Lee et al., 2007). Furthermore, TNFα also activates MTORC1 by PKB-dependent stimulation of IKKα, that associates with MTOR, inducing its activity (Dan et al., 2007).

The canonical Wnt pathway also plays a role in MTORC1 activation. When activated, this pathway inhibits the glycogen synthase kinase 3β (GSK3β) that phosphorylates and activates TSC2 (Inoki et al., 2006). GSK3β is also inhibited by PKB and RSK (Patel et al., 2004).

Several stress conditions interfere with MTORC1 activity. For instance, the stress triggered by DNA damage results in activation of the TSC complex, in a P53-dependent manner, with concomitant inhibition of MTORC1 signalling (Feng et al., 2005). Additionally, hypoxia and endoplasmic reticulum stress are characterized by an intricate interplay with MTORC1 signalling, which will be addressed in detail.

III.3.1.1.1. MTORC1 and Endoplasmic Reticulum (ER) stress

The ER is a membrane-bound organelle with a key role in cell growth and homeostasis due to its involvement in lipid and protein biosynthesis as well as in calcium storage, protein folding, maturation and transport. When ER function is perturbed namely by aberrant rates of protein synthesis or misfolding, by alterations in the calcium storage, oxidative stress or alterations in the redox equilibrium, an integrated response composed of three branches, collectively known as unfolded protein response (UPR), is triggered. The signalling proteins responsible for the activation of each branch are the inositiol-requiring protein 1 (IRE1), the PERK and the ATF6, respectively (reviewed in Ron and Walter, 2007).

The primary outcome of UPR comprises the reduction of protein influx into the ER and an increment in the efficiency of its folding capacity, which is accomplished by reduction of protein production and translocation into the ER, in conjunction with an increase in expression of genes involved in protein folding. When those actions fail to restore ER function, the UPR elicits cell death. Upon ER stress, an unusual function of IRE1 is activated, encompassing the endonucleolytic cleavage and subsequent splicing of the transcript encoding X-box binding protein 1 (XBP1). The spliced XBP1 transcriptionally induces the expression of a myriad of genes to amplify the ER folding capacity, including those encoding ER chaperones, and proteins involved in ER biogenesis, ER-associated protein degradation, phospholipid production and secretion (Lee et al., 2003). This UPR branch triggers an apoptotic route via activation of the apoptosis signal-regulating kinase-1 (ASK1) and its target c-Jun N-terminal kinase (JNK), through IRE-1 binding to the adaptor protein TNF receptor-associated factor-2 (Nishitoh et al., 2002; Urano et al., 2000). It has been demonstrated that PKB inhibits the IRE1-JNK axis (Kato et al., 2011). IRE1 also triggers the so called regulated IRE1-

dependent decay pathway, which induce degradation of several membrane-bound mRNAs (Hollien et al., 2009). The second branch of UPR is triggered by activation of PERK that phosphorylates EIF2α leading to overall protein synthesis reduction (Harding et al., 1999; Harding et al., 2000a, 2000b). This allows reduction of protein influx into the ER lumen which could accentuate the stress condition and disable a proper recovery (Harding et al., 1999). On the other hand, increased EIF2α phosphorylation levels allows the selective translation of *ATF4* which, in turn, induces expression of proteins involved in ER redox control, such as the endoplamic reticulum oxidoreduction 1, and glucose metabolism, such as the glucokinase (Harding et al., 2000b, 2003; Lu et al., 2004; Yoshizawa et al., 2009). In addition, ATF4 induces CCAAT/enhancer-binding protein homologous protein (CHOP), a key player of ER stress-triggered cell death (Harding et al., 2000b; Marciniak et al., 2004) that, in turn, regulates several proteins, such as the pro-apoptotic growth arrest and DNA damage-inducible protein-34 (GADD34), a specific phosphatase of phosphorylated EIF2α (Marciniak et al., 2004; Novoa et al., 2001). Apoptosis is further induced by increased EIF2α phosphorylation through downregulation of the anti-apoptotic myeloid cell leukemia sequence 1 (BCL2-related) (MCL-1) protein (Allagnat et al., 2011). The PERK branch is inhibited by the PI3K/PKB signalling pathway (Mounir et al., 2011). The third branch of UPR is triggered by the ATF6, which induces the expression of ER chaperones and proteins involved in membrane biogenesis (Bommiasamy et al., 2009; Haze et al., 1999, 2001; Yamamoto et al., 2007).

The fact that UPR and MTORC1 are involved in similar biological processes, such as lipid synthesis and angiogenesis (reviewed in Appenzeller-Herzog and Hall, 2012) might anticipate an interplay between them. In fact, the link between MTORC1 signalling and UPR has a role in development of several pathological conditions, like in type 2

diabetes (Bachar et al., 2009), and it has been demonstrated that MTORC1 regulates and is regulated by UPR (reviewed in Appenzeller-Herzog and Hall, 2012). The effects of MTORC1 activation on increasing the overall protein production could be enough to trigger UPR due to ER overloading of proteins. However, it seems that the history is more complicated, and the players of this network are becoming known. The ATF6-triggered UPR branch induces not only PKB (Yamazaki et al., 2009) but also RHEB (Schewe and Aguirre-Ghiso, 2008), which are both positive regulators of MTORC1 activity, as previously mentioned. In turn, activated MTORC1 induces UPR and, although it seems that chronic activation of MTORC1 might affect all UPR branches (Kang et al., 2010; Kato et al., 2011, 2013; Ozcan et al., 2008), the IRE1-JNK axis is deemed to be the main target of MTORC1. By activating IRE1-JNK, MTORC1 assists ER stress-induced apoptosis (Kato et al., 2011, 2013; Ozcan et al., 2008) and it has been demonstrated that this induction occurs via suppression of the PKB inhibitory role towards the IRE1-triggered branch (Kato et al., 2011). This inhibition of PKB might be assisted by CHOP, as it mediates the induction of the tribbles homolog 3, which is an PKB inhibitor (Du et al., 2003; Ohoka et al., 2005). CHOP and MTORC1-mediated inhibition of PKB might liberate the PKB-dependent inactivation of PERK-EIF2α (Mounir et al., 2011) and might account for the inhibition of MTORC1 activity observed upon prolonged ER stress (Deldicque et al., 2011; Nakajima et al., 2011; Di Nardo et al., 2009). This inhibition of MTORC1 increases autophagic death of ER-stressed cells (Qin et al., 2010).

III.3.1.1.2. **MTORC1 in Hypoxia**

Oxygen is essential for proper cell function and, in hypoxia, cells trigger a program that relies mainly in the action of HIFs. HIFs function as heterodimers consisting of an

oxygen- insensitive β subunit and one of three isoforms of the oxygen-sensitive α subunit, HIF1α, HIF2α and HIF3α (reviewed in Majmundar et al., 2010). The HIF1α isoform is expressed in all the tissues, whereas HIF2α and HIF3α isoforms expression is confined to certain cells (Bertout et al., 2008). Upon hypoxic or anoxic conditions, a burst in the expression of the low abundant HIF1α protein isoform occurs, as well as its dimerization with HIFβ subunit (Wang et al., 1995). HIF dimers associate with co-factors to form a functional transcriptional complex that binds and regulates transcription of a variety of genes harboring hypoxia response elements (Manalo et al., 2005; Pawlus and Hu, 2013; Semenza et al., 1996). Those include genes involved in angiogenesis, glucose transport, redox homeostasis and lipid metabolism (reviewed in Majmundar et al., 2010). Additionally to this transcriptional-mediated gene expression reconfiguration, a translational reprogramming is also observed. Low oxygen availability causes EIF4E association with 4ET (that inhibits EIF4F complex formation), PERK-mediated EIF2α phosphorylation by UPR induction, in addition to inhibition of the eukaryotic elongation factor 2 (EEF2) and MTORC1 signalling inhibition (Arsham, 2003; Koritzinsky et al., 2006, 2007; Koumenis et al., 2002; Liu et al., 2006). Those events result in overall translation inhibition and selective translation of a subset of transcripts (reviewed in Koritzinsky and Wouters, 2007). Inhibition of MTORC1 signalling inhibition in hypoxia occurs through several mechanisms, as illustrated in Figure I.4. Lack of oxygen restrains mitochondrial oxidative phosphorylation thus reducing the intracellular ATP levels with concomitant AMPK activation (reviewed in Kemp et al., 1999). According to its role in MTORC1 signalling regulation (as mentioned previously), it has been demonstrated that AMPK-mediated activation of TSC2 inhibits MTORC1 in hypoxic conditions, in a manner that is independent on HIF1α activity (Liu et al., 2006).

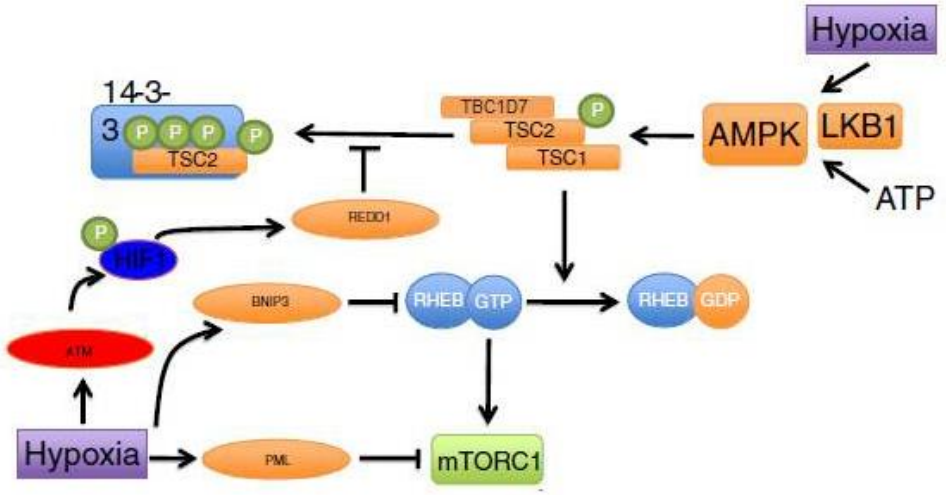


Figure I.4. MTORC1 regulation by hypoxic conditions. Hypoxia results in AMP-activated protein kinase (AMPK) activation and increased transcription of regulated in development and DNA damage responses 1 (REDD1) both inhibiting MTORC1 through activation of the TSC complex. In addition, the positive effect of RHEB towards MTORC1 is inhibited by the Bcl2/adenovirus E1B 19 kDA protein-interacting protein 3 (BNIP3) that binds and inhibits RHEB; and by promyelocytic leukemia (PML) that prevents subcellular colocatization of MTORC1 with RHEB. *Adapted with permission from Cam and Houghton, 2011.*

On the other hand, ataxia telangiectasia (ATM)- phosphorylated and stabilized HIF1α transcriptionally induces expression of regulated in development and DNA damage responses 1 (REDD1) which activates the TSC complex, by disruption of its binding with 14-3-3 leading to stabilization of the TSC1-TSC2 interaction (Brugarolas et al., 2004; Cam et al., 2010; DeYoung et al., 2008; Reiling and Hafen, 2004; Vega-Rubin-de-Celis et al., 2010). In addition, HIF1α also induces transcription of Bcl2/adenovirus E1B 19 kDA protein-interacting protein 3 (BNIP3) that binds and inhibits RHEB (Li et al., 2007). Furthermore, in hypoxic conditions, the promyelocytic leukemia (PML) protein induces MTOR nuclear accumulation, thus inhibiting its co-localization with RHEB (Bernardi et al., 2006) (Figure I.4). Furthermore, it has been suggested that MTORC1 activity is also inhibited by hypoxia through direct inhibition of the MTORC1 complex in a manner that requires a heme containing protein of unknown identity (Tan and Hagen, 2013).

In some experimental settings, MTORC1 signalling is inhibited shortly after the hypoxic insult whereas in others this inhibition occurs latter (Arsham, 2003; Koritzinsky et al., 2006). Probably the intensity of the hypoxic stimuli and combination with other stress signals might determine this variability. Actually, it has been suggested that EIF2α phosphorylation and EIF4F dissociation are triggered sequentially in severe hypoxic conditions. In that model, the protein synthesis reduction observed in the acute response to severe hypoxia is PERK-EIF2α-mediated, whereas the chronic response is mediated by MTOR inhibition and EIF4E sequestration by 4ET (Koritzinsky et al., 2006).

On the other hand, the interplay between MTORC1 signalling and hypoxia is bidirectional, as HIF1α expression is particularly affected by this signalling pathway as well as it transcriptional activity. MTORC1 induces *HIF1α* translation, in a manner that is highly dependent on *HIF1α* 5´UTR (Bernardi et al., 2006; Laughner et al., 2001; Thomas et al., 2006). A 5´ terminal oligopyrimidine tract (TOP) element within *HIF1α* 5´UTR is partially responsible for this dependency of *HIF1α* mRNA translation on MTORC1 signalling (Thomas et al., 2006). Furthermore, the RHEB-MTOR axis induces HIF1α transcriptional activity by MTORC1-HIF1α interaction through RAPTOR, that promotes HIF1α interaction with its co-activator CBP/p300 (Hudson et al., 2002; Land and Tee, 2007; Zhong et al., 2000).

The main substrates of MTORC1 complex are components of the translational apparatus; accordingly a particular emphasis will be given to the role of this complex in the regulation of protein synthesis.

III.3.1.1.3. **MTORC1 translation regulation**

MTORC1 complex regulates directly or indirectly several components of the translational apparatus. The direct targets of phosphorylation are 4EBPs and S6 kinases (S6Ks) (Blommaart et al., 1995; Hara et al., 1998). 4EBPs are a family of proteins comprising 4EBP1, 4EBP2 and 4EBP3, while there are two mammalian S6K isoforms, S6K1 and S6K2 (Lin et al., 1994, 1994; Poulin et al., 1998; Shima et al., 1998) . Since the vast majority of reports are referring to 4EBP1 and S6K1, here the data referring to those proteins will be presented.

MTORC1 directly binds to the mRNP complex and EIF3 is the platform for MTORC1 activity on the regulation mRNA translation initiation (Holz et al., 2005). In the absence of a stimulatory signal, EIF3 binds to the unphosphorylated S6K1 forming a free EIF3-S6K1 complex. Upon stimulation, MTORC1 binds to this complex and this interaction triggers MTORC1-mediated S6K1 phosphorylation and its release from the EIF3-MTORC1 complex. In addition, MTORC1 directs the EIF3 complex to the cap structure to phosphorylate 4EBP1 which prompts its release from EIF4E (Harris et al., 2006; Holz et al., 2005). The free S6K1 is fully activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1)-mediated phosphorylation (Alessi et al., 1997) and targets several substrates that assist mRNA translation.

4EBP1 is involved in the regulation of EIF4F complex formation, as mentioned before. Upon MTORC1 activation, the hyper-phosphorylated 4EBP1 does not bind to EIF4E, allowing EIF4G binding and thus, EIF4F complex formation (Beretta et al., 1996; Brunn et al., 1997; Burnett et al., 1998; Haghighat et al., 1995; Hara et al., 1997; Lin et al., 1994; Mader et al., 1995; Marcotrigiano et al., 1999; Pause et al., 1994). It has been demonstrated that 4EBP1 is re-phosphorylated by MTORC1 upon prolonged treatment with rapamycin, and this re-phosphorylation is accompanied by derepression

of cap-dependent translation, despite the maintenance of S6K1 dephosphorylation (Choo et al., 2008). This is in agreement with the finding that 4EBP1 is the major effector of MTORC1 signalling in the regulation of mRNA translation (Hsieh et al., 2012; Thoreen et al., 2012). Acute inhibition of MTORC1 leads to reduction of mRNA translation, with a special effect on a subset of mRNAs that are highly dependent on 4EBP1. The 5′UTRs of those mRNAs have either 5′TOPs, 5′ TOP-like motifs or pyrimidine-rich translational elements (PRTE). A 5′TOP consist of a 5′ terminal cytidine followed by 4 to 14 consecutive pyrimidines (reviewed in Meyuhas, 2000), whereas as 5′ TOP-like motifs have at least 5 consecutive pyrimidines and or PRTE comprise a uridine at position +6 flanked by pyrimidines (Hsieh et al., 2012; Thoreen et al., 2012). Translation of mRNAs containing IRES elements seems to be refractory, or even augmented by MTORC1 inhibition. mRNA translation of those TOP and TOP-like mRNAs have a special requirement for EIF4G, as the EIF4E-cap interaction is assisted by this initiation factor, and MTORC1 inhibition disrupts the EIF4G-EIF4E interaction in a 4EBP-dependent manner (Hsieh et al., 2012; Thoreen et al., 2012). In addition, EIF4G is phosphorylated in a rapamycin dependent-manner (Raught et al., 2000), though the direct effects of MTOR-mediated EIF4G phosphorylation in mRNA translation has not been addressed.

Previously to the discovery of 4EBP1 as a master regulator of MTORC1-mediated mRNA translational control, especially of TOP and PRTE mRNAs, it was assumed that the other MTORC1 phosphorylation-target, S6K1 (Burnett et al., 1998), was determinant for efficient translation of transcripts with TOP elements (Jefferies et al., 1994; Schwab et al., 1999). In particular, it was assumed that the effector protein responsible for this action of S6K1 was its phosphorylation-target ribosomal protein S6 (RPS6) (reviewed in Hornstein et al., 2001). Nevertheless, this assumption was rapidly

changed upon the observation that TOP mRNAs are translated even upon deletion of S6K1 and concomitant RPS6 unphophorylation (Stolovich et al., 2002; Tang et al., 2001). S6K1 phosphorylates EIF4B and PDCD4 with stimulation of protein synthesis (Dennis et al., 2012; Dorrello et al., 2006; Raught et al., 2004; Shahbazian et al., 2006). S6K-mediated phosphorylation of EIF4B is required for the assembly of this initiation factor into PIC (Holz et al., 2005); whereas PDCD4 phosphorylation reverts its inhibitory effect towards EIF4A (Yang et al., 2003). Newly synthesized transcripts undergo a pioneer round of translation, an important point for mRNA quality control, that differs from the steady-state translation namely because the cap structure is bound to the cap-binding protein 80 (CBP80) and CBP20 (Chiu et al., 2004). It has been found that the S6K1 Aly/REF-like target (SKAR), a phosphorylation target of S6K1 and regulator of cell growth (Richardson et al., 2004), interacts both with the exon-junction complex (EJC) of CBP80-bound mRNAs and with activated S6K. The EJC is a multisubunit complex deposited ~20 nt upstream of each exon-exon junction during splicing (Le Hir et al., 2000). These SKAR interactions are fundamental to recruit S6K1 to newly synthesized mRNAs, and the SKAR-S6K axis assists the increment in translation of spliced mRNAs (Ma et al., 2008). The cap-binding protein CBP80 itself is phosphorylated by S6K1 at sites which are phosphorylated also by growth factor stimulation (Wilson et al., 2000b). However, it has been suggested that S6K1 is not a fundamental player, by itself, in the mRNA translation rate, as rapamycin-induced inactivation of S6K1 has a low negative impact on ribosomal occupancy of translating mRNAs in mouse embryonic fibroblast cells (Thoreen et al., 2012). Actually, it was recently suggested that S6K1 has a particular role in assuring translation fidelity rather than rate, possibly by affecting the ribosome speed during translation elongation (Conn and Qian, 2013). The fact that the S6K-RPS6 axis is involved in the production

of ribosomal proteins, by transcriptional induction, in conjunction with the fact that the MTOR-S6K1 axis activates the EEF2, a fundamental player of the mRNA translation elongation step, might account for those observations (Chauvin et al., 2013; Proud et al., 2001). Accordingly, a synergistic effect between S6K1 and the master regulator 4EBP1 seems to be fundamental in the control of mRNA translation (Dennis et al., 2012).

In addition, MTORC1 also regulates mRNA translation by augmenting the expression of other components of the translational apparatus. MTORC1 increases the expression of rRNA by activating, in a S6K1-dependent manner, the tripartite motif-containing protein-2A and inducing its interaction with RNA Pol I (Mayer et al., 2004), whose activation is also induced by S6K1 (Hannan et al., 2003). In addition, MTORC1 induces transcription of 5S rRNA and tRNA by reversing the inhibitory effect of Maf1 towards Pol III (Kantidakis et al., 2010; Shor et al., 2010).

III.3.1.2. **MTOR complex 2**

MTORC2 activity is important for actin cytoskeleton reorganization, protein synthesis and maturation, cell survival migration and metabolism (reviewed in Oh and Jacinto, 2011). MTORC2 is unresponsive to acute treatment with rapamycin but its activity is modulated by growth factors, such as insulin, that stimulates the interaction of MTORC2 with ribosomes, via the PI3K signalling pathway, and this interaction seems to be required for MTORC2 activation (Figure I.3) (Zinzalla et al., 2011). The role of nutrients or amino acids in MTORC2 regulation has been subjected to intensive debate, although it is deemed that leucine has a role in MTORC2 signalling, since addition of this amino acid to starved cells induces MTORC2-dependent cell migration (Hernández-Negrete et al., 2007). In contrast to MTORC1, the TSC complex

positively regulates MTORC2 signalling in a manner that is independent on its GAP activity towards RHEB (Huang et al., 2008).

The MTORC2 signalling activates, by phosphorylation, different members of the AGC kinase family, such as the pro-survival serum- and glucocorticoid-induced protein kinase 1 (SGK1) (García-Martínez and Alessi, 2008). In addition, PKB is also activated by MTORC2, through phosphorylation either at Ser473 or Thr450 (Facchinetti et al., 2008; Hresko and Mueckler, 2005; Ikenoue et al., 2008; Sarbassov et al., 2006), in a growth factor-sensitive or insensitive manner, respectively (Alessi et al., 1996; Bellacosa et al., 1998; Hauge et al., 2007). MTORC2-mediated activation of PKB is repressed by S6K1-mediated phosphorylation of RICTOR (Dibble et al., 2009). Furthermore, the protein kinase C isotypes, a family of proteins involved in cytoskeletal function, are phosphorylated by MTORC2 contributing to their maturation and stability (Ikenoue et al., 2008).

III.3.2. **MTOR inhibition by rapamycin**

The action of rapamycin on MTOR relies on its ability to bind to FK-506 binding protein of 12 kDa (FKB12), an intracellular receptor, which tightly binds to the FKBP12-rapamycin binding (FRB) specific domain of MTOR (Brown et al., 1994; Choi et al., 1996; Sabatini et al., 1994). Binding of the FKB12-bound rapamycin to MTOR precludes the accessibility of substrates to its catalytic center (Yang et al., 2013). In addition, upon prolonged exposure to this macrolide, the RAPTOR-MTOR binding is inhibited (Oshiro et al., 2004), possibly by a physical constraint imposed by the MTOR-bound rapamycin/FKB12 complex (Yang et al., 2013). It has been observed that, in opposition to MTORC1 that is readily inhibited by rapamycin, only prolonged or high doses of this macrolide has an action on MTORC2, in cell type-dependent manner

(Jacinto et al., 2004; Sarbassov et al., 2004, 2006). It has been suggested that the components of MTORC2 physically inhibit the access of the rapamycin/FKBP12 to its binding site on FRB (Yang et al., 2013) and a prolonged treatment might be effective due to inhibition of RICTOR and MSIN1 binding to the rapamycin/FKB12 complex-bound MTOR (Sarbassov et al., 2006; Yang et al., 2013). Furthermore, it has been demonstrated that rapamycin differently affects the MTORC1 substrates: whereas S6K is efficiently dephosphorylated upon rapamycin treatment, 4EBP1 is only moderately affected (Thoreen et al., 2009). In addition, the response of these substrates to prolonged exposure to rapamycin is different: while S6K dephosphorylation is maintained, 4EBP1 regains its phosphorylation in an MTORC1-dependent fashion (Choo et al., 2008).

III.3.3. MTOR hyperactivation and the need for its inhibition

MTOR is hyperactivated in several diseases including benign and malignant tumors. Among the several biological activities in which MTOR signalling participates, it has been suggested that its role in promoting protein synthesis is of fundamental importance in tumor development, by inducing expression of anti-apoptotic, angiogenic and glycolytic genes, as well as genes involved in cell cycle regulation (reviewed in Laplante and Sabatini, 2012). Studies addressing the overall targets of MTORC1-mediated translational regulation showed a particular bias towards transcripts encoding proteins involved in protein synthesis, cell invasion and metastization (Hsieh et al., 2012; Thoreen et al., 2012). Besides its role in inducing mRNA translation, MTORC1 signalling further contributes to tumorigenesis by promoting lipid biosynthesis, important for membrane formation; stimulates the pentose phosphatase pathway, important for nucleotide synthesis and redox control; inhibits autophagy, that might

contribute to tumor cell survival or tumor development (reviewed in Laplante and Sabatini, 2012). The MTORC2 signalling is also involved in tumor development, namely by its role in regulating PKB, that is frequently deregulated in cancer (reviewed in Tokunaga et al., 2008).

Several MTOR inhibitors, including rapalogs (rapamycin analogs), have been developed, showing good results in clinical trials, although with lower efficacy than expected (reviewed in Pópulo et al., 2012). The fact that MTORC1 signalling has rapamycin-resistant roles (Choo et al., 2008; Thoreen et al., 2009), triggers negative feedback-loops, namely leading to PKB activation, in combination with the fact that MTORC2 signalling also activates PKB (reviewed in Huang and Manning, 2009), anticipate the poor capacity of rapalogs to reverse MTOR tumorigenic potential. Accordingly, two other classes of MTOR inhibitors were developed and are being used in clinical trials for several tumor types, the MTOR kinase inhibitors and dual PI3K/MTOR kinase inhibitors (reviewed in Don and Zeng et al., 2011), The MTOR kinase inhibitors block its catalytic activity by competition with ATP and have the advantage to predictably impair both complexes and inhibit the MTORC1 rapamycin-resistant functions (Feldman et al., 2009). Nevertheless, it has been demonstrated that some of these inhibitors, such as Torin 1, efficiently impairs proliferation and diminishes cell size through total abolishment of MTORC1 functions but in a manner that is independent of MTORC2 (Thoreen et al., 2009). However, other ATP-competitive inhibitors, such as INK128, are effective in targeting both MTORC1 and MTORC2 signalling pathways (Janes et al., 2013). It has been shown that this compound impairs prostate cancer development by triggering apoptosis and impairing cell proliferation, invasion and metastasis (Hsieh et al., 2012).

It has been reported that, although MTOR signalling inhibition is cytostatic, in some cases, enhanced survival of tumor cells is observed (reviewed in Easton and Houghton, 2006; Faivre et al., 2006). This has been attributed mainly to MTORC1-triggered activation of the PI3K pathway, leading to enhanced cell survival (reviewed in Rosen and She, 2006). Accordingly, dual PI3K/MTOR kinase inhibitors have been developed and promising results have arisen from its use in clinical trials (reviewed in Pópulo et al., 2012). Regarding to the fundamental role of MTORC1 and MTORC2 in important biological processes, it remains to be determined the effects of long-term inhibition of MTOR signals.

In addition to cancer, MTOR deregulation is also associated with other diseases, such as metabolic diseases, neurodegenerative disorders and autoimmune diseases (reviewed in Dazert and Hall, 2011).

III.4. P53

P53 was discovered in 1979 in a context of studying tumor development by SV40 infection. Different groups realized that a cellular protein, with a molecular mass of 53kDa, coprecipitated with the SV40 large T-antigen in cells infected with this virus (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979; Smith et al., 1979). In parallel, it was observed that cells transformed by the Abelson murine leukemia virus also produced the same protein (Rotter et al., 1980). Furthermore, it was shown that the humoral response of immunized mice with non-viral transformed cells encompassed production of antibodies for this 53kDa protein (DeLeo et al., 1979). The refereed protein was named "P53" according to its molecular mass, which was estimated on basis of its migration in SDS-PAGE. However, it was found latter that the correct molecular mass of human P53 protein is actually

43.7 kDa and that the misconclusion derived probably from the presence of a high number of proline residues altering migration of P53 (or shall we say P43.7?).

Initially and during some years, it was believed that P53 was an oncogene rather than a tumor suppressor. This assumption arose from many apparent compelling experimental evidences. It formed complexes with an oncogenic protein (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Melero et al., 1979; Smith et al., 1979) and its expression was enhanced in primary mouse tumors in opposition to normal thymocytes (Rotter, 1983). In addition, many of the studies addressing the oncogenic potential of P53 were erroneously performed with mutant instead of wild-type *TP53*, due to erroneously cloning of *TP53* cDNA sequences deriving from cancer cells and, thus, carrying mutations. As expected, overexpression of those mutant P53 proteins contributed to tumour growth (Parada et al., 1984). The true story of P53 as a *bona fide* tumor suppressor instead started when the wild-type sequence deriving from normal tissues was established (Eliyahu et al., 1988; Finlay et al., 1988). The hallmarks for the recognition of *TP53* as tumor suppressor gene were the following observations: 1. Loss of wild-type *TP53* alleles in colorectal carcinomas by mutations and/or deletions (Baker et al., 1989); 2. Suppression of MYC and RAS-induced transformation by overexpression of wild-type *TP53* (Eliyahu et al., 1989). 3. *TP53* germline mutations contribute to the hereditary Li-Fraumeni syndrome (Malkin et al., 1990) and 4. *TP53* knockout mice are prone to cancer (Donehower et al., 1992). With the emergency of several studies reporting the protective roles of P53, it turned from the “bad guy” to the "guardian of the genome".

III.4.1. P53 function

P53 is a transcription factor of the P53 family, which is additionally composed of P63 and P73 proteins that share structural, biochemical and biological similarities (reviewed in Dotsch et al., 2010). This protein has a pivotal role in suppressing tumor development through inhibition of cell proliferation and growth, by inducing temporary or permanent cell cycle arrest and by promoting cell death (reviewed in Zilfou and Lowe, 2009). Upon an abnormal cellular signal such as activation of oncogenes, DNA damage, loss of cell-cell contact and hypoxia, P53 either develops a protecting response allowing cells to bypass the adverse condition and survive, by inducing cell cycle arrest, triggering a DNA repair response, regulating the response to metabolic stress and promoting an antioxidant response; or triggers a program that ultimately leads to senescence, apoptosis or autophagic cell death (reviewed in Vousden and Prives, 2009). The protective or destructive outcome of P53 activation is determined mainly by the type and severity of the stress condition or cellular damage (reviewed in Bensaad and Vousden, 2007). Although it seems clear that both P53-triggered responses are homeostatic and tumor unfriendly, some pro-survival functions of P53 may also assist tumorigenesis, when deregulated. In addition, its anti-survival functions might also contribute to premature aging (reviewed in Vousden and Prives, 2009).

The response of P53 relies mainly on its ability to modulate expression, at the transcriptional level, of a myriad of genes (reviewed in Riley et al., 2008). P53 controls transcription through direct binding, as a tetramer, to a consensus site that contains two copies of the sequence RRRCA/TT/AGYYY separated by up to 13 bps (el-Deiry et al., 1992; Funk et al., 1992). Those P53 response elements *(*P53RE) are located mainly within the promoter and/or intronic region of P53-targets, that include genes involved in cell cycle arrest, apoptosis, DNA repair, transcription, metabolism, cell adhesion and

motility and membrane function (reviewed in Riley et al., 2008). Upon different stimuli, P53 regulates transcription of different genes and this selection is mainly determined by the P53REs and P53 protein levels. Genes with strong P53REs recruit P53 even when this protein is present in low levels (in unstressed or low-stressed cells), whereas increased P53 protein levels allow binding to weaker P53 consensus sites. The former encompasses genes required for cell cycle regulation while the latter comprises pro-apoptotic genes (Weinberg et al., 2005). The selectivity of P53 towards different genes is also determined by stress-triggered phosphorylation, acetylation and methylation events as long as by several P53 binding partners (reviewed in Gu and Zhu, 2012; Vousden and Prives, 2009).

III.4.1.1. Pro-apoptotic role of P53

A master role of P53 in preventing tumor development is the elimination of cells with transforming potential through induction of apoptosis. The apoptotic process occurs through the intrinsic or extrinsic pathways. In the intrinsic pathway the apoptotic stimuli, such as DNA damage, ER stress or oncogene activation, activates BCL2 homology 3 (BH3)-only proteins that, in turn, stimulate BCL2-associated X protein (BAX) and BCL2-antagonist/killer (BAK) and lead to mitochondrial permeabilization. Subsequently, several apoptogenic factors are released from the mitochondria, such as cytochrome *c*, which triggers activation of the caspase cascade. The extrinsic pathway is induced by ligand binding to cell death receptors, which triggers the recruitment of adaptor molecules leading to activation of caspases 8 and 10 that, in turn, activates effector caspases. The effectors of both pathways are the caspases 3, 6 and 7 that cleave several cellular components ultimately leading to formation of apoptotic bodies (reviewed in Portt et al., 2011). The intrinsic pathway is regulated by P53, through

induction of the BH3-only protein members P53-upregulated modulator of apoptosis (PUMA) (Nakano and Vousden, 2001), phorbol-12-myristate-13-acetate-induced protein (PMAIP1) (Oda et al., 2000), and BH3 interacting-domain death agonist (BID) (Sax et al., 2002) as well as of BAX (Miyashita et al., 1994). It also induces the expression of APAF1 (Kannan et al., 2001; Moroni et al., 2001; Robles et al., 2001), to which the cytoplasmic relocated-cytochrome *c* binds, triggering formation of the apoptosome that activates the initiator caspase 9 (Jiang and Wang, 2000). The calcium-binding protein PDCD6, that induces cytochrome *c* release, is also activated by P53 (Suzuki et al., 2012). P53 regulates the extrinsic pathway namey by targeting the death receptor 5 (*DR5*) (Wu et al., 1997). In addition, P53 also induces the effector caspase 6 (MacLachlan and El-Deiry, 2002).

P53 also targets genes whose products are involved in the production of reactive oxygen species (ROS), whose accumulation is important for the apoptotic response triggered by this transcription factor (Johnson et al., 1996; Li et al., 1999). Additionally, P53 regulates several microRNAs that regulate expression of a myriad of genes involved in the apoptotic response (Chang et al., 2007). For instance, the miR-34s family, that is involved in temporary and permanent cell cycle arrest and cell death induction, namely by targeting *BCL2,* is transcriptionally regulated by P53 (Bommer et al., 2007). Moreover, P53 also triggers apoptosis in a transcriptional-independent manner. Following stress, P53 can be redistributed into the mitochondria where it inhibits the anti-apoptotic proteins BCL2L1 and BCL2 (Mihara et al., 2003).

III.4.1.2. Pro-survival role of P53

One of the major causes of P53-mediated apoptosis is an irreversible damage on DNA (Roos and Kaina, 2006). Before committing an aberrant cell to the apoptotic route, P53 imposes a cell cycle arrest and manages a program to ensure proper damage repair. This "second chance" is given to low stressed-cells harboring manageable injuries. A key player in P53-mediated inhibition of cell cycle progression is the CDKN1A (el-Deiry et al., 1993; Harper et al., 1993). *CDKN1A* is transcriptional activated by P53 upon mild stress conditions or even in normal conditions when P53 protein levels are low. The potent repressing activity of CDKN1A towards the G1/S transition regulators CDK 2, 3, 4 and 6, avoids cell cycle progression into S phase (el-Deiry et al., 1993; Harper et al., 1993, 1995).The P53-mediated cell cycle arrest at G2 is namely achieved by transcriptional activation of *14-3-3σ* and *GADD45* (Hermeking et al., 1997; Laronga et al., 2000; Zhan et al., 1999), that inactivate the cyclin B/CDK1 complex (Laronga et al., 2000; Zhan et al., 1999). Activation of this complex is fundamental for entry into mitosis (reviewed in Lindqvist et al., 2009).

The protective and pro-survival role of P53 relies also in the activation of a program that assists DNA repair. Besides its intrinsic ability to repair DNA lesions, P53 also induces the expression of genes involved in this process, such as MutL homolog 1 (*MLH1*), damage-specific DNA binding protein 2 (*DDB2*) and *GADD34*, that are involved in mismatch repair, nucleotide excision repair and *de novo* DNA synthesis, respectively (Chen and Sadowski, 2005; Smith et al., 1994; Tan and Chu, 2002; reviewed in Gatz and Wiesmüller, 2006).

III.4.1.3. Tumor suppressor activities of P53

The tumor suppressor activities of P53 are not confined to its ability to induce apoptosis and temporary cell cycle arrest, as this transcription factor also senses and regulates metabolic changes, triggers autophagy, induces senescence, elicits antioxidant and antiangiogenic responses and limits cell invasion.

A role for P53 at inhibiting hyper-activated MTORC1 has been demonstrated. The activated P53-targets sestrin1 and sestrin2 induce AMPK-dependent phosphorylation of TSC2, leading to MTORC1 inhibition (Budanov and Karin, 2008). Other inhibitors of the MTOR signalling are also activated by P53, such as phosphatase and tensin homolog (PTEN) and insulin-like growth factor binding protein 3 (IGFBP3) (Feng et al., 2007). This inhibition has a role in the reversibility of the P53-mediated cell cycle arrest (Korotchkina et al., 2010; Leontieva et al., 2010, 2011). The inhibition of the MTOR pathway constitutes also one way by which P53 induces autophagy (Feng et al., 2005). Another way to induce autophagy is via activation of the damage-regulated autophagy modulator (DRAM), that leads to macroautophagy (Crighton et al., 2006). Autophagy triggered by P53 either contributes to increased cell survival or elicits cell death (Amaravadi et al., 2007; Crighton et al., 2006). Other functions of P53 have been ascribed to its role in the regulation of metabolism, such as decrease of glucose uptake (Kawauchi et al., 2008; Schwartzenberg-Bar-Yoseph et al., 2004), and promotion of mitochondrial respiration (Matoba et al., 2006), thus impairing the Warburg effect, a metabolic change of cancer cells towards energy production through aerobic glycolysis rather than oxidative phosphorylation (Warburg, 1956).

P53 displays the ability to counteract aberrant cells also by inducing permanent cell cycle arrest, or senescence, of transforming and malignant cells (Chen et al., 2005; Schmitt et al., 2002; Serrano et al., 1997). The main effector of P53-mediated

senescence is CDKN1A and a role for the plasminogen activator inhibitor 1 (PAI1) has also been observed (Brown et al., 1997; Kortlever et al., 2006; Leal et al., 2008).

P53 also develops an antioxidant response, through induction of several antioxidant genes that restrain the intracellular levels of reactive oxygen species thereby preventing DNA damage and genome instability (Liu et al., 2008; Sablina et al., 2005).

Another important function of P53 at restraining tumor development is its antiangiogenenic action, namely by activating inhibitors of neovascularization and endothelial cell growth, such as the brain-specific angiogenesis inhibitor 1 and the collagen-4 prolyl hydroxylase (Nishimori et al., 1997; Teodoro et al., 2006).

Furthermore, the invasive ability of a tumor cell is also constrained by P53, since it targets genes involved in restraining cell migration, EMT, filopodia formation and extracellular matrix degradation (Chang et al., 2011; Gadéa et al., 2002; Kunz et al., 1995; Mashimo et al., 1998; Yuan et al., 2013).

III.4.2. P53 protein and isoform counterparts

The *TP53* gene encodes twelve different proteins derived by alternative promoter usage, splicing and initiation of translation. Those P53 products display a crucial role in the P53 activity and its functions are dictated mainly by the P53 protein domains that are retained or lost (reviewed in Olivares-Illana and Faahraeus, 2010). Accordingly, the structure of P53 protein will be addressed first.

III.4.2.1. Structure of P53 protein

The human *TP53* gene is located at chromosome 17p13.1 (McBride et al., 1986 and Isobe et al., 1986), spans 20 kb, contains 11 exons and two promoters: a distal (P1) and an internal (P2) within intron 4.

The human P53 protein has a subset of domains each corresponding to specific functions. Furthermore, it contains five areas of high conservation across species, which are termed conserved boxes. The N-terminal of P53 contains two acidic transactivation domains, TAD I and II, corresponding to amino acids 1-40 and 43-63, respectively (Venot et al., 1999). TAD I and II can function independently or in cooperation to activate transcription (Candau et al., 1997). TAD I comprises the conserved box I located at amino acids 15-29, which is required for binding of the mouse double minute 2 homolog (MDM2), an important regulator of P53 (Chen et al., 1993; reviewed in Manfredi, 2010). A proline-rich domain (PXXP) is located at amino acids 63-91 and it influences transactivation of some P53-target genes (Venot et al., 1998; Edwards et al., 2003). The DNA binding domain (DBD), that binds to the P53REs, spans amino acids 102 to 300 and contains the remaining conserved boxes (el-Deiry et al., 1992). The oligomerization domain (OD) spans amino acids 325 to 356 comprising a nuclear export signal (NES) (amino acids 340 to 351). The P53 complexes into a tetramer via its OD to become a functional transcription factor (Clore et al., 1995). The major nuclear localization signal (NLS) is located between amino acids 305 and 322. The NES and NLS regulate protein travelling from and into the nucleus (Shaulsky et al., 1990). The C-terminus of P53 contains a regulatory domain (amino acids 365-393) and two less active NLSs (366- 372 and 377-381). A nonspecific DNA-binding domain lies between amino acids 364 and 390, which downregulates the binding capacity of the central DBD (Bayle et al., 1995).

III.4.2.2. **P53 isoforms**

Transcription from *TP53* gene at each promoter originates six isoforms, which can be divided into two sets, depending on the mRNA translation initiation site. *TP53* gene is

also subjected to alternative splicing of intron 9, generating three sets of isoforms with different C-terminal domains, α, β and γ. The α isoforms, which derives from complete exclusion of intron 9, contain the classical P53 C-terminal OD, whereas the β and γ isoforms are generated by partial retention of intron 9, resulting in substitution of the OD by 10 or 15 new amino acids, respectively (reviewed in Olivares-Illana and Faahraeus, 2010). Transcription from *TP53* gene at the distal promoter originates three isoforms derived by mRNA translation initiation at the main methionine (codon 1), P53, P53β and P53γ and three other that internally initiate at codon 40, Δ40P53α, Δ40P53β and Δ40P53γ. *TP53* gene transcribed from the intragenic promoter originates the *Δ133P53* transcript leading to the formation of the isoforms Δ133P53α, Δ133P53β, Δ133P53γ and Δ160P53α, Δ160P53β, Δ160P53γ which are a result of initiation at codons 133 or 160, respectively (reviewed in Khoury et al., 2009). Those isoforms lack TAD I and II and part of DBD (Marcel et al., 2010a).

The P53β and γ isoforms regulate P53 transcriptional activity though via distinct mechanisms. P53β isoform forms complexes with P53 and can either inhibit or increase P53 transcriptional activity. In addition, it has P53-independent transcriptional activity in P53REs (Bourdon et al., 2005; Fujita et al., 2009). It has been demonstrated that P53β isoform induces apoptosis in a P53-independent manner and enhances apoptosis trigerred by P53 (Bourdon et al., 2005). The P53γ protein isoform activates the internal promoter of *TP53* gene, in a P53-independent manner, and regulates P53 transcriptional activity on the *BAX* promoter (Bourdon et al., 2005).

Δ40P53 protein isoforms are generated by alternative splicing of intron 2 (Ghosh et al., 2004) or internal translation, mediated by an IRES element, at codon 40 (Candeias et al., 2006; Ray et al., 2006). Δ40P53 lacks TAD I, and the Mdm2-binding site (Yan et al., 2002) but retains OD, DBD and TAD II. Accordingly, it has been shown that Δ40P53

form complexes with P53, modulating its activity and stability; and the content of Δ40P53 protein isoform on P53-Δ40P53 hybrid complexes is determinant on this modulatory role (Candeias et al., 2006; Courtois et al., 2002; Ghosh et al., 2004; Powell et al., 2008; Yin et al., 2002). A role for Δ40P53 in counteracting the growth suppressor activity of P53 has been observed (Courtois et al., 2002; Ghosh et al., 2004). Its transcriptional activity differs from that of P53, as exemplified by its preferential induction of 14-3-3σ upon ER stress (Bourougaa et al., 2010). In those settings, synthesis of Δ40P53 is stimulated in PERK-dependent fashion and the induction Δ40P53 homo-oligomerization promotes G2 arrest without affecting G1 progression. In these stress conditions, Δ40P53 is also able to trigger apoptosis in a P53-independent manner (Bourougaa et al., 2010).

Δ133P53α isoform modulates the cellular outcome in response to DNA damage and developmental defects and has a role in promoting cell proliferation (Aoubala et al., 2011; Chen et al., 2009; Fujita et al., 2009). Its expression is regulated by P53 itself upon genotoxic stress, by transactivation of the internal promoter (Aoubala et al., 2010; Marcel et al., 2010a). P53 binds P53REs via its L1 loop of the DBD, which is absent in Δ133P53α protein isoform. Accordingly, Δ133P53α is unable to bind P53REs in a P53-independent manner (Marcel et al., 2010a). Nevertheless, it has been shown that it complexes with P53 (Aoubala et al., 2010) and inhibits P53-P53RE binding (Marcel et al., 2010a), suggesting that the P53-Δ133P53α hybrid complex has an altered ability to regulate transcription. In fact, P53 transcriptional activity on *CDKN1A* promoter is inhibited by Δ133P53α (Bourdon et al., 2005). By reconfiguring the ability of P53 to regulate gene expression, Δ133P53α protein inhibits P53-mediated apoptosis (Aoubala et al., 2010; Bourdon et al., 2005; Chen et al., 2009). The Δ133P53α and γ protein isoforms stimulate angiogenesis, in opposition to Δ133P53β; and it seems that

the function of the two latter isoforms does not rely on their ability to affect P53 transcriptional activity (Bernard et al., 2013; Bourdon et al., 2005).

The *Δ133P53* transcripts also generate the Δ160P53α, β and γ protein isoforms through initiation at codon 160 (Marcel et al., 2010b). Both Δ160P53α and β localize in the nucleus, similarly to Δ133P53α and β, but whilst Δ160P53α is perinuclear, Δ160P53β presents a foci pattern (Marcel et al., 2010b). The isoform Δ160P53γ was not experimentally identified until now. The functions of Δ160P53 isoforms are largely unknown, although a role for Δ160P53β in erythrocyte differentiation has been suggested as it is downregulated by hemin treatment (Marcel et al., 2010b).

Additional splice variants were discovered in ovarian cancer cells, P53ΔE6 and P53δ, P53ε and P53ζ, arising from alternative splicing due to somatic mutations at splice sites of introns 6 and 9 (Hofstetter et al., 2010). However, little is known about theirs functions.

III.4.2.2.1. P53 isoforms and cancer

A role for P53 isoforms in tumorigenesis has been discovered. For instance, expression of P53β protein isoform in ovarian cancer cells is associated with poor overall survival when functional P53 is also present (Hofstetter et al., 2010). In those settings, aberrant expression of this P53 protein product is caused by mutations at splice sites of intron 9 of *TP53* gene (Hofstetter et al., 2010), which indicate that silent mutations at *TP53* gene affect expression of the P53 isoforms. Furthermore, Δ40P53 is overexpressed in mucinous ovarian cancer (Hofstetter et al., 2012) and it has been suggested that it is involved in melanoma development (Avery-Kiejda et al., 2008). Overexpression of the murine homolog of Δ133P53α (Δ122p53α) in mice causes enhanced cell proliferation, decreased apoptosis and tumor development (Slatter et al., 2011). Furthermore,

Δ133P53 in conjunction with P53β seems to be involved in adenoma-to-carcinoma transition, as the expression of Δ133P53α is reduced and P53β is increased in senescent colon adenomas, a tendency that is reversed in colon carcinomas (Fujita et al., 2009). The role and expression pattern of Δ160P53 isoform in cancer is unknown, although it is interesting to note that roughly 4% of all somatic mutations predicted to disrupt P53 coding sequence corresponding to the N-terminal region do not affect the sequence encoding Δ160P53 (Petitjean et al., 2007).

III.4.3. IRES-mediated control of P53

The *TP53* transcript harbors two IRESs elements that regulate production of P53 full-length protein and the N-terminal truncated protein isoform Δ40P53. These IRESs are located in *TP53* 5´UTR and coding region between AUG1 and AUG40, respectively (Candeias et al., 2006; Ray et al., 2006; Yang et al., 2006). The activity of each IRES element influence the other, in a way that the IRES for Δ40P53 also allows mRNA translation initiation at AUG1 and P53 IRES inhibits Δ40P53 protein synthesis (Candeias et al., 2006; Yang et al., 2006). Furthermore, the Y-shaped structure of the IRES for P53 positions the AUG in a hairpin domain that comprises also part of *TP53* coding region (Błaszczyk and Ciesiołka, 2011; Grover et al.,, 2011), suggesting a role for this region in the activity of the IRES element. Actually, it has been demonstrated that the natural structure of *TP53* 5´UTR requires a sequence downstream of the initiation codon (Błaszczyk and Ciesiołka, 2011). A distinct regulation of both IRESs elements is observed during cell cycle. Whereas the IRES element that induces cap-independent production of P53 is mainly active in the G2/M transition, the IRES for Δ40P53 is mostly functional in the G1/S transition (Ray et al., 2006). Furthermore, a cell type- and cellular stress–specificity of activation of each *TP53* IRES element is also

observed and this differential activation plays an important role in P53 activity. The IRES for P53 is activated by ectoposide-induced DNA damage, during OIS and is slightly augmented upon ER stress, while the IRES for Δ40P53 is mostly stimulated upon ER stress and serum starvation (Candeias et al., 2006; Grover et al., 2009; Weingarten-Gabbay et al., 2013; Yang et al., 2006). Upon ER stress, the Δ40P53 IRES is stimulated by PERK and the induced Δ40P53 protein isoform stimulates transcription of *14-3-3σ* and concomitant G2 arrest. In those settings, not only P53 has no impact on G2 arrest but also the P53-mediated G1 arrest is suppressed by Δ40P53. The dominancy of function of Δ40P53 over P53 is deemed to be caused not only by increased expression of the former protein but also by ER stress-induced formation of transcriptionally active Δ40P53 homotetramers (Bourougaa et al., 2010). This differential activation might be attributed to ITAFs that differently modulate the activity of each IRES element, as it occurs with the recently identified P53 ITAF death-associated protein 5 (DAP5), a member of the EIF4G family that retains EIF4G central domain, but lacks the N-terminal domain that binds to the cap-binding protein EIF4E (Imataka et al., 1997; Levy-Strumpf et al., 1997; Shaughnessy et al., 1997; Weingarten-Gabbay et al., 2013; Yamanaka et al., 1997). It has been demonstrated that although DAP5 binds with similar affinities to P53 IRES and Δ40P53 IRES, it specifically assists the IRES-mediated translation of the latter, both in stress and unstressed condition, affecting the induction of the Δ40P53-preferential target *14-3-3σ*. The modulatory activity of DAP5 on P53 IRES is restricted to few cellular conditions, suggesting that its action on this IRES element requires additional factors (Weingarten-Gabbay et al., 2013). Actually, several proteins bind to *TP53* 5′UTR (Takagi et al., 2005) and a combinatorial action of different ITAFs might be required for efficient IRES-mediated synthesis of P53. Indeed, it has been suggested that the interconnection between PTB,

PTB associated splicing factor (PSF/SFPQ) and Annexin A2 ITAFs might be important for the IRES-mediated translation of *TP53* transcript (Sharathchandra et al., 2012). The modulatory effect of PTB is induced by its cytoplasmic translocation triggered upon DNA damage conditions (Grover et al., 2008; Sharathchandra et al., 2012). Curiously, PSF/SFPQ has been associated with cancer cell proliferation; and Annexin A2 is involved in cell motility, migration and invasion (Tsukahara et al., 2013; Zhai et al., 2011). Other ITAFs modulating the IRES elements located in the *TP53* transcript have been identified. For instance, a role for dyskerin in promoting IRES-mediated production of P53 has been demonstrated and mutations in the *DKC1* gene lead to impaired IRES-mediated synthesis of P53 (Bellodi et al., 2010; Montanaro et al., 2010). Furthermore, the RPL13a also plays a role in the IRES-mediated ribosomal recruitment to the *TP53* transcript (Chaudhuri et al., 2007). Other proteins influence *TP53* mRNA translation, but the exact mechanism and involvement in IRES-mediated translation has not been directly assessed. An example is the stimulatory role of hnRNP Q on *TP53* mRNA translation through binding to its 5´UTR (Kim et al., 2013). Furthermore, nucleolin and RPL26 also regulate *TP53* mRNA translation. Both proteins interact with a double-stranded RNA domain of *TP53* mRNA, formed by 5´UTR-3´UTR base-pairing; and differently regulate *TP53* translation: whereas nucleolin has a repressor role in *TP53* mRNA translation, RPL26 stimulates it (Chen et al., 2012; Chen and Kastan, 2010; Takagi et al., 2005). It has been suggested that the RPL26-mediated *TP53* mRNA translation induction upon stress conditions is caused by disruption of *TP53* mRNA-bound nucleolin dimers (Chen et al., 2012). Whether RPL26 and nucleolin proteins or the 5´UTR-3´UTR hybrid RNA domain have a role on *TP53* IRES elements has to be determined. Furthermore, MDM2 binds to an RNA hairpin domain immediately downstream of *TP53* initiation codon and stimulates translation of *TP53*

(Błaszczyk and Ciesiołka, 2011; Candeias et al., 2008). Upon genotoxic stress, MDM2 is redistributed to the nucleus and this subcellular localization is mandatory to incite *TP53* mRNA translation (Gajjar et al., 2012). This suggests that MDM2 might be an ITAF of the *TP53* IRESs. Last but not least, P53 protein itself binds to 5´UTR of *TP53* mRNA and negatively regulates its expression (Fontoura et al., 1997; Mosner et al., 1995; Yin et al., 2003).

These observations demonstrate that IRES-mediated translation has a key role in modulating P53 activity. The differential transcriptional targets of Δ40P53 and its dominancy, in some conditions, over P53 leading to inhibition of its growth suppression role, clearly establish a role for IRES-dependent translation in the tumor suppressor activity of P53. In addition to that, it has been demonstrated that silent mutations or single-nt polymorphisms found in human tumors decrease IRES-mediated translation of *TP53* mRNA (Grover et al., 2011; Khan et al., 2012).

IV. AIMS

IRESs elements assist protein synthesis upon translational inhibitory conditions and have been reported in transcripts encoding proteins involved in stress responses, oncogenes and tumor suppressor genes (reviewed in Sonenberg and Hinnebusch, 2007). The principal aim of this work was to investigate the IRES-mediated translational control of *MTOR* and *Δ133P53* transcripts. MTOR signalling displays fundamental roles in cellular homeostasis and its deregulation is associated with numerous pathological conditions (reviewed in Laplante and Sabatini, 2012). The mechanisms regulating MTORC1 and MTORC2 signals as well as their downstream effects have been subjected to intensive research, in opposition to regulation of *MTOR* expression itself. The fact that MTOR signalling pathway is active upon translational inhibitory conditions (Schewe and Aguirre-Ghiso, 2008), and the immediate reactivation of inhibited MTORC1 upon a stimulatory signal (Hara et al., 1998; Tan and Hagen, 2013), prompted me to study whether *MTOR* expression is regulated at the translational level. The specific aims to address this question were to:

i) Investigate whether human *MTOR* transcript has an IRES element;

ii) Test if cellular stress conditions, in which MTOR signalling plays a role, affect the activity of this IRES element;

iii) Analyze the activation status of *MTOR* IRES upon MTORC1 inactivation;

iv) Check if *MTOR* IRES is affected by inhibition of two important translational control points.

TP53, one of the most common mutated genes in cancer (reviewed in Rivlin et al., 2011), governs the expression of several protein isoforms (reviewed in Khoury and Bourdon, 2009). The mechanism responsible for the expression of the majority of P53 protein products is well known, in opposition to the N-terminal truncated Δ160P53

protein isoform. The fact that this isoform arises from internal initiation at codon 160 of the *Δ133P53* transcript (Marcel et al., 2010b), prompted me to study whether a cap-independent mechanism is governing its expression. The specific aims to achieve this goal were to:

i) Analyze the Δ160P53 protein expression in stress conditions that are known to inhibit cap-dependent translation;

ii) Test if an IRES element within *Δ133P53* transcript is regulating Δ160P53 protein expression;

iii) Investigate the effect of stress conditions in the activity of this IRES element;

iv) Address how is this IRES element regulated.

V. MATERIALS AND METHODS

V.1. Plasmid constructs

The dicistronic vector carrying Renilla luciferase (RLuc) and Firefly luciferase (FLuc) ORFs was based on the psiRF vector (Tahiri-Alaoui et al., 2009). A splice donor site within RLuc ORF was changed by site-directed mutagenesis, using primers #1 e #2 (Table 1). The resulting construct was named pR_Fhp-. In order to prevent ribosome reinitiation, a sequence of a stable hairpin was PCR amplified with primers #3, #4 (Table 1) from plasmid P53 "A" (Candeias et al., 2006), digested with XhoI and cloned into pR_Fhp-. The resulting construct was named pR_hp_F (refereed as pR_F, for simplicity). The same hairpin was PCR amplified with primers #5 and #6 (Table 1) from plasmid P53 "C" (Candeias et al., 2006) and cloned into pCR@2.1 TOPO (Life Technologies), generating the GFPhp_TOPO construct. The GFPhp_TOPO construct was digested with AgeI and HindII and the originated overhangs were filled-in using Quick Blunting™ Kit (New England Biolabs), originating the hp_TOPO construct. The hp_TOPO construct was subsequently digested with NheI and the resultant fragment was cloned into the pR_Fhp- vector. The resulting construct was named phpR_F. Human β-globin (*HBB*) 5'UTR and *MTOR* 5'UTR (NM_004958.3) were PCR amplified, using primers #7-#8 and #9-#10, respectively (Table 1). In parallel, pR_F vector was amplified with primers #11-#13 and #12-#13 for *HBB* and *MTOR* 5´UTR, respectively. The respective fragments were subjected to SOEing PCR with primers #7-#13 and #9-#13 for *HBB* and *MTOR* 5'UTR, respectively (Table 1). The resultant PCR products were digested with XmaI/AccI or NotI/BsrGI, for *HBB* and *MTOR* 5'UTRs, respectively, and cloned into pR_F, generating the pR_HBB_F and pR_mTOR_F constructs, respectively. The same strategy was used for cloning *MLH1* 5'UTR, but with primers

#14 to #17 (Table 1) and the enzymes XmaI/BsrGI; the resultant construct was called pR_MLH1_F. In a similar manner, two putative IRESs sequences (258 and 432-nt long) of Δ160P53 were PCR amplified using primers #18-#19 and #18-#20, respectively. In parallel, pR_F vector was amplified with primers #21-#13 and #22-#13, respectively (Table 1). The respective fragments were subjected to SOEing PCR with primers #18-#13 and the resultant PCR products were digested with NotI/BsrGI and cloned into pR_F. The generated constructs were called pR_ Δ160nt258_F and pR_Δ160nt432_F, respectively. The same strategy used for generating the pR_Δ160nt432_F construct was applied for cloning the 432-nt long sequence proceeded by the putative Δ160p53 5´UTR, only with the exception that primer #23 was used instead of #18 (Table 1). The resultant construct was called pR_5´Δ160432_F. The human *MYC* and EMCV IRESs sequences were PCR amplified from MYC-IRES-Ova and EMCV-IRES-Ova plasmids (Apcher et al., 2008), using primers #24-#25 and #26- #27 for MYC and EMCV IRES, respectively (Table 1). In parallel, the pR_Fhp- vector was amplified with primers #28-#17 and #29-#17 for *MYC* and EMCV IRES, respectively (Table 1). SOEing PCR was performed with the resultant PCR products using primers #24-#17 and #26-#17 for *MYC* and EMCV IRES, respectively (Table 1). The generated fragments were digested with EcoRI/AccI and cloned into psimutR_F, creating psimutR_MYC_F and psimutR_EMCV_F plasmids, respectively. To generate pR_c-myc_F and pR_EMCV_F, the previous plasmids were digested with XmaI/BsrGI and cloned into pR_F vector. To generate the promoterless constructs, pR_F was digested with NheI/BglII, blunt-ended with Quick Blunting Kit (New England Biolabs) and re-ligated, originating the promoterless_pR_F plasmid. pR_mTOR_F and pR_MLH1_F plasmids were digested with EcoRV/BsrGI and the resultant fragments were cloned into promoterless_pR_F,

originating the promoterless_pR_mTOR_F and promoterless_pR_MLH1_F, respectively.

The remaining constructs were performed by Marco Candeias (Kyoto University, Japan).

Table III.1 DNA oligonucleotides used in the current work.

Primer	Sequence
#1	CGCTCCAGATGAAATGGGCAAGTACATCAAGAGCTTC
#2	GAAGCTCTTGATGTACTTGCCCATTTCATCTGGAGCG
#3	CCGCTCGAGCGGGGTACCAATGACGCGCGC
#4	TCCCCCCGGGGGGATCATGGATCCTTTCGCGCG
#5	CTAGCTAGCTAGTCATGGATCCTTTCGCGCG
#6	CCCATTGACGCAAATGGGCGGTAGGCG
#7	TCCCCCCGGGGGGAACATTTGCTTCTGACACAAC
#8	CATCGGCCATGGTGTCTGTTTGAGGT
#9	ATAAGAATGCGGCCGCTAAACTAGCTCCCGGCTTAGAGGACA
#10	ATCGGCCATCTTGCCCTGAGGTTCGCG
#11	ACAGACACCATGGCCGATGCTAAGAACA
#12	CAGGGCAAGATGGCCGATGCTAAGAACATT
#13	GTGAGAGAAGCGCACACAG
#14	TCCCCCGGGGGAGAAGAGACCCAGCAACCCAC
#15	TAGCATCGGCCATTTTGGCGCCAGAAGAGC
#16	GGCGCCAAAATGGCCGATGCTAAGAACA
#17	GCAAATCAGGTAGCCCAGG
#18	ATAAGAAAGCGGCCGCTAAACTATGGCCATCTACAAGCAGTC
#19	GCATCGGCCATGCCGCCCATGCAGGA
#20	CATCGGCCATGCTCCCTGGGGGCAGCT
#21	GGCGGCATGGCCGATGCTAAGAACA
#22	CAGGGAGCATGGCCGATGCTAAGAACA
#23	ATAAGAATGCGGCCGCTAAACTATTTTTGCCAACTGGCCAAGAC
#24	GGAATTCCAATTCCAGCGAGAGGCAGAG
#25	TAGCATCGGCCATCGTCTAAGCAGCTGCAAGGAGA
#26	GGAATTCCAATTCCGCCCCTCTCCCTCCCC
#27	TAGCATCGGCCATTTATCATCGTGTTTTTCAAAGG
#28	GCAGCTGCTTAGACGATGGCCGATGCTAAGAACA
#29	CGATGATAAATGGCCGATGCTAAGAACA
#30	GTCTCGAACTTAAGCTGCAG
#31	CGAAGTACTCGGCATAGGTG
#32	GGACGCTCCAGATGAAATGG
#33	TTACACGGCGATCTTGCCG

III.2. Cell culture and plasmid transfection

HEK293T, HeLa, A549, Sw480, HCT116, H1299 and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) while NCM460 cells were maintained in

Roswell Park Memorial Institute medium (RPMI) 1640, both supplemented with 10% (v/v) fetal bovine serum. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. Transient transfection was performed using Lipofectamine 2000 or 293fectin (Invitrogen), according to manufacturer's instructions, in 35-mm plates and, then, harvested after the indicated times. H1299 cells were transfected with 300ng of each plasmid. To assay FLuc and RLuc activities, 1µg (HEK293T, Sw480 and H1299), 1.5µg (HeLa), 2ug (NCM460) or 2.5µg (A549 and HCT116) of each dicistronic plasmid were transfected. For the experiments involving drug treatments for the MTOR analysis, HEK293T cells were transiently transfected with 0.5µg of each dicistronic DNA. For the promoterless assays and to test hairpin efficiency at inhibiting ribosome scanning, cells were co-transfected with a plasmid encoding β-galactosidase (β-gal) and the dicistronic plasmids (β-gal:dicistronic) as follows: 0.5µg:0.5µg for HEK293T cells; 1.5µg:1.5µg for HeLa cells and 2µg:1µg for A549 cells. To mimic hypoxia, 2 hours post-transfection, HEK293T cells were changed to fresh medium supplemented with 100µM or 200µM $CoCl_2$ (Sigma-Aldrich), or vehicle (Vh) (H_20) during 24 hours. To induce ER stress, 19 hours post-transfection, HEK293T, A549 or H1299 cells were treated with 1µM or 0.25µM thapsigargin (TG) (Sigma-Aldrich) or Vh (DMSO) during 20 hours. Alternatively, H1299 cells were treated, 24 hours post-transfection, with 12µM tunicamycin (TU) (Sigma-Aldrich) or Vh (DMSO), alone or in combination with 10µg/mL cyclohexamide (CHX) or 25µM MG132 for 2 hours. Untransfected A549 and HCT116 cells were treated with 1µM TG, 12µM TU or Vh (DMSO) during 20 hours. To inactivate MTORC1, 14 hours post-transfection, HEK293T cells were treated with 80nM rapamycin (Rap) (Sigma) or drug Vh (DMSO) during 6 hours. For stress induced by cell over-confluency, A549, NIH3T3 and H1299 cells were grown during 2-10 days

to achieve overconfluency, or collected at a density of 300cells/mL (normal cell density).

III.3. *In vitro* transcription and RNA transfection

pR_F e pR_mTOR_F plasmids were linearized at the 3' end using ClaI, *in vitro* transcribed, capped and poly-adenylated with mMessage mMachine T7 kit (Ambion) and poly(A) tailing kit (Ambion), respectively, according to manufacturer's instructions. RNA samples were treated with Turbo DNase (Ambion) and purified by phenol:chloroform extraction. Transcripts quality was analyzed by denaturing formaldehyde-agarose gel electrophoresis. HEK293T cells were transfected with 1µg of each RNA, using Lipofectamine 2000 (Invitrogen) and luciferase activity was assayed 4 hours post-transfection.

III.4. RNA isolation

Total RNA from transfected cells was prepared using Nucleospin RNA extraction II (Marcherey-Nagel) followed by treatment with RNase-free DNase I (Ambion) and purification by phenol:chloroform extraction.

III.5. Reverse transcription-PCR

First strand cDNA synthesis from 1µg of total RNA was carried out using SuperScript II Reverse Transcriptase (Life Technologies) and oligod(T) primer, according to the manufacturer's standard protocol. cDNAs were PCR amplified using primers #30 and #31 (Table III.1) for fragment I, or #32 and #33 (Table III.1) for fragment II. To control for DNA contamination, PCR reactions were also carried out without prior

cDNA synthesis. Samples were analyzed by electrophoresis on 0.8% agarose gels. The resulting fragments were then gel-purified and sequenced.

III.6. Luciferase assays

Cell lysis was performed with Passive Lysis Buffer (Promega) and then cells were subjected to a freeze-thaw cycle at -80°C to 37°C and centrifuged at maximum speed for 5 minutes. The cell lysates were used to determine luciferase activity with the Dual-Luciferase Reporter Assay System (Promega) and a Lucy 2 luminometer (Anthos Labtec), according to the manufacturer's standard protocol. Ten µL of cell lysate were assayed for FLuc and RLuc enzymatic activities. Ratio is the unit of FLuc after normalized to RLuc, and each value was derived from three independent experiments.

III.7. Sodium dodecyl sulphate - polyacrilamide gel electrophoresis (SDS-PAGE) and immunoblot

Protein lysates were resolved, according to standard protocols, in 12, 10 or 8% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were probed using rabbit polyclonal anti-MTOR (Sigma) at 1:1500 dilution, mouse monoclonal anti-HIF1α (BD Biosciences) at 1:750 dilution, rabbit monoclonal anti-PARP (Cell Signalling) at 1:750 dilution, rabbit monoclonal anti-Phospho-p70 S6K (Thr389) (Cell Signalling) at 1:1000 dilution, rabbit monoclonal anti-p70 S6K (Cell Signalling) at 1:750 dilution, rabbit polyclonal anti-Phospho-EIF2α (Ser52) (Invitrogen) at 1:750 dilution, rabbit polyclonal anti-EIF2α (Cell Signalling) at 1:500 dilution, rabbit polyclonal CM1 (a gift from Borek Vojtesek, Masaryk Memorial Cancer Institute, Czech Republic) at 1:8,000 dilution, mouse monoclonal anti-α-tubulin (Sigma) at 1:10,000 dilution or mouse monoclonal anti-PCNA (Calbiochem) at 1:1000 dilution. Detection was carried out

using secondary peroxidase-conjugated anti-mouse IgG (Bio-Rad) or anti-rabbit IgG (Bio-Rad) antibodies followed by chemiluminescence.

III.8. Statistical analysis

Data are presented as means ± standard deviation of at least three independent experiments. Test F was used for evaluation of variances equality. Student's two-tailed *t*-test was used for estimation of statistical significance. Significance for statistical analysis was defined as $p<0.05$.

III.9. RNA folding prediction

The MFold program (http://mfold.rna.albany.edu/?q=mfold) was used for prediction of RNA secondary structure. This prediction is based on the thermodynamic stability using an empirically determined energy table. Stability of the predicted secondary structure is inversely correlated with the estimated energy (Mathews et al., 1999). The following constraints were applied: free energy increment was set to 10%. As a first approach, three *MTOR* RNA segments were analyzed: nt 1 to 50, nt 1 to 121 (full-length 5'UTR) and nt 1 to 171 (full-length 5'UTR and a portion of *MTOR* coding region). As a second approach, the analysis was expanded to the following sequences: nt 1-221 and 1-271. The most stable secondary structure predicted for each segment was compared to the others and the conserved elements (loops, stems, hairpins, etc.) were considered.

IV. RESULTS

Marco Candeias (University of Kyoto, Japan) performed the work presented at Figures Figures IV.8 and IV.9. Rafaela Lacerda and Alexandre Teixeira (Instituto Nacional de Saúde Dr. Ricardo Lisboa, Portugal) performed the *in vitro* transcription of pR_F and pR_MTOR_F constructs (RNAs used in Figure IV.3).

IV.1. IRES-dependent translational regulation of *MTOR*

IV.1.1. Human *MTOR* 5'UTR has IRES classical features

Some transcripts have translational advantage in adverse conditions through regulatory elements within their 5'UTRs, such as upstream uORFs and/or IRESs that allow or facilitate their translation independent of the availability of ternary and/or EIF4F complexes (reviewed in Sonenberg and Hinnebusch, 2007). Indeed, these elements have been identified in a number of eukaryotic mRNAs encoding proteins involved, namely, in signal transduction pathways, gene expression regulation, apoptosis or/and stress responses (reviewed in Sonenberg and Hinnebusch, 2007). The fact that MTOR is a key player of a signalling pathway involved in several cellular functions (reviewed in Laplante and Sabatini, 2012), prompted us to examine whether the 5'UTR of human *MTOR* mRNA has uORF and/or IRES features. The alignment of human *MTOR* 5'UTR with that of chimpanzee, mouse, chicken, turkey and rat revealed relatively conserved sequences, especially those in close proximity to the AUG codon (Figure IV.1A), which may indicate the existence of a regulatory element. Further inspection revealed that human *MTOR* 5´UTR does not contain any uAUG. On the other hand, fifteen potential non-AUG initiators were found, five of which fulfilled the requirement of a purine at position -3 and a "G" at position +4 (Kozak, 1991) (Table IV.1). Since the

severe dependence of non-AUG initiation codons on an optimal Kozak context may rely also on the entities of other surrounding nucleotides (Chen et al., 2008; Kozak, 1989a, 1991), the sequence context of those five potential initiators was compared with Kozak consensus sequence and the results showed that none of them matched perfectly. It has been demonstrated that hairpins located 11 and mainly 17-nts downstream of the start codon enhance translation at initiation codons with poor Kozak context (Kozak, 1990). Accordingly, the AUG_hairpin program (Kochetov et al., 2007) was used for prediction of hairpins at those positions and no eligible downstream hairpins for any of the five selected non-AUG codons (Table IV.1) were predicted. Thus, it is unlikely that *MTOR* 5´UTR contains a functional uORF. In contrast, it has a high GC-content (74%) and is 121-nts long, being longer than the stated dogma of the "usually < 100 nt" median (reviewed in Baird, 2006). Analysis of *MTOR* 5'UTR sequence with M-Fold program (Zuker, 2003) showed that its predicted folding has a Gibb's free energy of -55.50 kJ/mol, is Y-shaped and contains a stem-loop from nt 4 to 25 (SL I; Figure IV.1B). This stem-loop is conserved in the resultant folding of nt 1 to 50, nt 1 to 121 (full-length 5'UTR) and nt 1 to 171 (full-length 5'UTR and a portion of *MTOR* coding region) of the *MTOR* mRNA. The relative conservation of *MTOR* 5'UTR and its IRES-like features suggests that *MTOR* translation might be regulated *via* an IRES element.

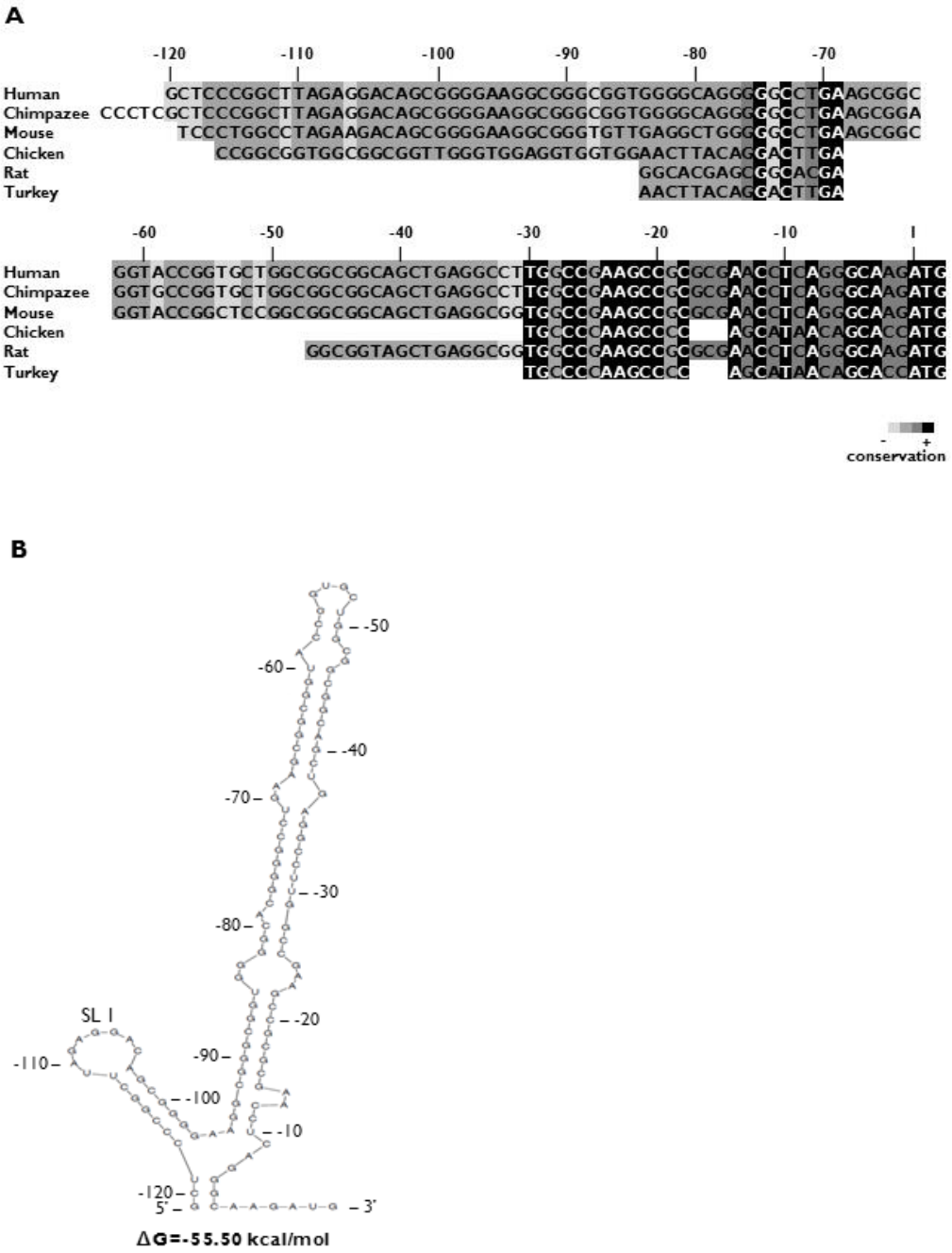


Figure VI.1. *MTOR* 5'UTR has IRES classical features. (A) Nucleotide sequence alignment of the human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), mouse (*Mus musculus*), chicken (*Gallus gallus*), rat (*Rattus norvegicus*) and turkey (*Meleagris gallopavo*) *MTOR* mRNA 5'UTRs shows two segments highly conserved among different species. Highly conserved nucleotides are shown in black boxes and non-conserved sequences are shown in white boxes. Distance (in nucleotides) from the AUG initiation codon is indicated above the sequence. **(B)** RNA secondary structure of human *MTOR* 5'UTR predicted by M-Fold program (http://mfold.rna.albany.edu/?q=mfold). A putative stem-loop structure (SL I) from nucleotides -98 to -118 (relatively to the AUG codon at position +1), nucleotides position, and the Gibb's free energy of the predicted secondary structure are indicated.

Table IV.1. None of the fifteen potential non-AUG initiation codons located at *MTOR* 5´UTR represents a bona fide translation initiator. The surrounding sequence of codons differing at a single nucleotide from AUG, located at *MTOR* 5´UTR, was analyzed in terms of Kozak consensus sequence match. The initiation context of five non-AUG codons fulfill the Kozak context requirements for a purine at position -3 and a "G" at position +4, but not for the nucleotides at the remaining positions. Analyses with AUG_hairpin program (http://wwwmgs.bionet.nsc.ru/mgs/programs/aug_hairpin/) show no eligible hairpins around nucleotides 11 and 17 downstream of the beginning of the coding sequence.

Potential non-AUG initiator	**Surruonding sequence**	**Kozak context match**		**Downstream stem-loop**
		Purine at position -3 and "G" at position +4	Nucleotides at the remaining positions	
GUG	CGG**G**CGGUG**G**	Yes	No	No
	GUA**C**CGGUG**C**	No	-	-
CUG	CCG**G**UGCUG**G**	Yes	No	No
	CGG**C**AGCUG**A**	No	-	-
	CGG**G**GCCUG**A**	No	-	-
UUG	GAG**G**CCUUG**G**	Yes	No	No
ACG	UGG**G**GCACG**G**	Yes	No	No
AAG	GCG**G**GGAAG**G**	Yes	No	No
	GGC**C**UGAAG**C**	No	-	-
	UGG**C**CGAAG**C**	No	-	-
	CAG**G**GCAAG**A**	No	-	-
AGG	GCU**U**AGAGG**A**	No	-	-
	CGG**G**GAAGG**C**	No	-	-
	CAG**C**UGAGG**C**	No	-	-
	AAC**C**UCAGG**G**	No	-	-

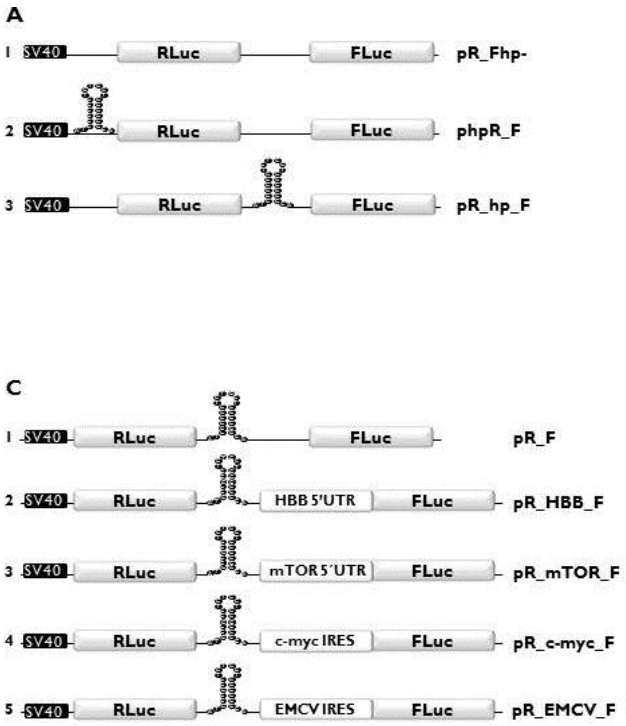
IV.1.2. *MTOR* 5'UTR supports Firefly luciferase activity in a dicistronic context with impaired reinitiation

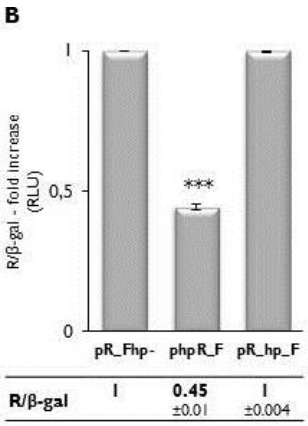
The most common method to test for IRES activity of a given sequence relies on DNA dicistronic reporter systems encoding mRNAs in which the upstream cistron is cap-dependent translated, whereas the downstream ORF is only translated if preceded by an IRES element. In this study, a dicistronic DNA reporter with Renilla luciferase (RLuc) ORF as the first cistron and Firefly luciferase (FLuc) ORF as the second cistron, was used. The multiple cloning site between both cistrons has no IRES activity (Tahiri-Alaoui et al., 2009). Since stable hairpins are effective at inhibiting ribosome scanning

(Kozak, 1986b), a hairpin structure was used in order to prevent translation reinitiation (Candeias et al., 2006). To confirm that the hairpin was effectively inhibiting ribosome scanning in our system, a stable hairpin was cloned upstream of RLuc, generating the phpR_F construct (Figure IV.2A) and the resultant RLuc activity from this construct was compared to that of a hairpin-less DNA dicistronic construct (pR_Fhp- from figure IV.2A). For that, HEK293T cells were transiently transfected with the aforementioned dicistronic DNA constructs along with a plasmid encoding β-galactosidase (β-gal), and cellular extracts were prepared and assayed for luciferase activity. RLuc activities were normalized to the activity of β-gal and the subsequent ratio of RLuc to β-gal (R/β-gal) was compared to that from the pR_Fhp- vector, arbitrarily set to 1 (Figure IV.2B). As seen in Figure IV.2B, insertion of the stem-loop upstream of RLuc effectively diminished its activity (~60%), demonstrating that it inhibits ribosome scanning, as previously shown (Candeias et al, 2006). By using a construct in which the hairpin is located upstream of FLuc ORF, instead (pR_hp_F from Figure IV.2A), it is possible to observe that this hairpin did not destabilizes RLuc activity, when located downstream of RLuc ORF (Figure IV.2B). Accordingly, the latter construct was used to test for IRES activity; and from now on it will be referred, for the sake of simplicity, as pR_F.

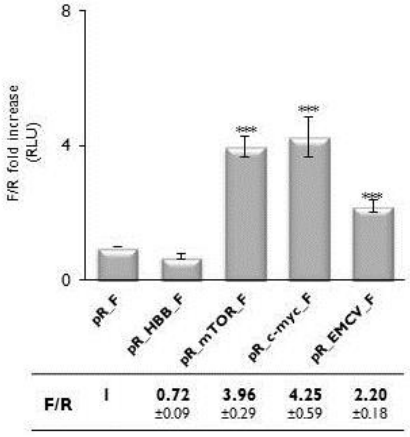
The *HBB* 5´UTR was used as a negative control for IRES activity, since its translation is exclusively cap-dependent (Lockard and Lane, 1978). The positive controls were *MYC* IRES (cellular IRES) (Stoneley et al., 1998), and EMCV IRES (viral IRES) (Jang et al., 1988). Human *MTOR* 5´UTR (NM_004958.3) and the aforementioned control sequences were cloned upstream of FLuc, in a way that the native initiation codon is replaced by the FLuc initiation codon. The resultant constructs were called pR_mTOR_F (*MTOR*), pR_HBB_F (*HBB*), pR_c-myc_F (*MYC*) and pR_EMCV_F

(EMCV). For normalization, the aforementioned empty reporter plasmid (pR_F) was used, which contains a short linker sequence between the hairpin and FLuc cistron (Figure IV.2C). Expression of each of these reporter genes was assessed after transient transfection into a panel of human cell lines – HEK293T, HeLa, and A549 cells and measurement of luciferase activity (Figure IV.2D-F). FLuc activity of each construct was normalized to the activity units from RLuc expressed in the same mRNA. The subsequent ratio between FLuc and RLuc (F/R) was compared to that from the empty pR_F vector, arbitrarily set to 1 (Figure IV.2D-F). Results showed that insertion of *MTOR* 5'UTR in the dicistronic reporter significantly enhanced relative production of FLuc: 4-fold in HEK293T cells, 3-fold in HeLa cells, and 2-fold in A549 cells. Besides, *HBB* 5'UTR did not induce relative FLuc activity, and *MYC* and EMCV IRESs were active in the three cell lines tested, as expected (Figure IV.2D-F). The *MTOR* 5'UTR-mediated relative FLuc activity was greater than the observed for EMCV IRES and similar to that of *MYC* IRES in HEK293T cells. In HeLa cells, relative FLuc activity driven by *MTOR* 5'UTR was about 60% of that driven by *MYC* IRES and 74% of that from EMCV IRES. On the other hand, in A549 cells, protein production from the second cistron through *MTOR* 5'UTR represented 40% of that through *MYC* IRES and was about 16% of the observed from EMCV IRES. Of note, in A549 cells, *MTOR* 5'UTR-mediated relative FLuc activity was similar to that observed for EMCV IRES in HEK293T cells, in which this IRES element is known to be active (Venkatesan et al., 2003). Together, these results show that *MTOR* 5'UTR allows production of FLuc in a dicistronic DNA reporter system with impaired translation reinitiation, strongly suggesting that this segment has IRES activity.

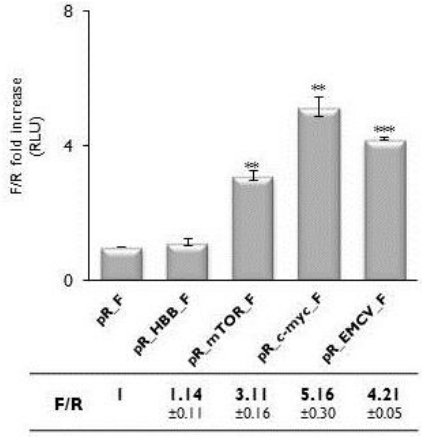




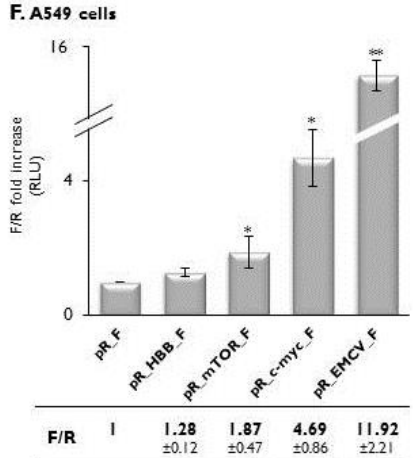
R/β-gal	1	0.45 ±0.01	1 ±0.004



F/R	1	0.72 ±0.09	3.96 ±0.29	4.25 ±0.59	2.20 ±0.18



F/R	1	1.14 ±0.11	3.11 ±0.16	5.16 ±0.30	4.21 ±0.05



F/R	1	1.28 ±0.12	1.87 ±0.47	4.69 ±0.86	11.92 ±2.21

Figure IV.2. *MTOR* 5'UTR supports Firefly luciferase (FLuc) activity in a dicistronic reporter DNA construct with impaired reinitiation (A) Scheme depicting the dicistronic reporter pR_Fhp-, phpR_F and pR_hp_F constructs. A stable hairpin was cloned upstream or downstream of the Renilla luciferase (RLuc) open reading frame (ORF) (RLuc box), generating the phpR_F and pR_hp_F constructs, respectively. The dicistronic transcriptional units expressing RLuc and Firefly luciferase (FLuc) are under the control of SV40 promoter. **(B)** The hairpin inhibits RLuc activity when located upstream but not when located downstream of RLuc ORF. HEK293T cells were transiently co-transfected with each of the constructs described in (A), along with a plasmid expressing β-galactosidase (β-gal). Luciferase activity was measured 16 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between RLuc and β-gal compared to that of the corresponding pR_Fhp- construct, which was arbitrarily set to 1. **(C)** Scheme depicting the dicistronic reporter pR_F (pR_hp_F from A), pR_HBB_F, pR_mTOR_F, pR_c-myc_F, pR_EMCV_F constructs. The 5' untranslated regions (5'UTRs) of *HBB* or of human *MTOR* mRNAs, the human *MYC* and the EMCV IRESs elements were cloned into the empty vector (pR_F), downstream of the RLuc ORF (RLuc box) and downstream of a stable hairpin structure, but upstream of the FLuc ORF (FLuc box), to create the pR_HBB_F, pR_mTOR_F, pR_c-myc_F, pR_EMCV_F constructs, respectively. **(D-F)** Relative enhancement of downstream reporter enzyme expression mediated by *HBB*, *MTOR*, *MYC* or EMCV segments, comparing to that from the empty construct, in HEK293T (D), HeLa (E), and A549 (F) cells. Cells were transiently transfected with the dicistronic plasmids depicted in (C) as indicated. Luciferase activity was measured 16 hours (HEK293T) or 24 hours (HeLa and A549) post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between FLuc and RLuc, normalized to that of the empty construct, which was arbitrarily set to 1. Data are presented below each graph as the means ± standard deviation (SD) of at least 3 independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$; (∗∗) $p<0.01$; (∗∗∗) $p<0.001$.

IV.1.3. *MTOR* 5´UTR-mediated FLuc expression is neither due to abnormal splicing nor promoter activation.

Cryptic promoter activity or abnormal splicing in the 5'UTR might generate a false-positive result if the generated monocistronic and/or aberrant dicistronic RNAs encode an enzymatically active FLuc. To test R_mTOR_F mRNA for abnormal splicing, a computer-assisted analysis for prediction of potential splice sites was performed with Splice View. This analysis revealed that *MTOR* 5´UTR does not contain any potential splice acceptor site. To confirm this prediction, the integrity of the RNA expressed in HEK293T cells from the pR_mTOR_F construct was analyzed by RT-PCR. As shown in Figure IV.3, only a full-length RNA is produced. Furthermore, the correspondent

PCR fragments were sequenced and no cryptic splicing was found (data not shown), indicating that the observed *MTOR* 5'UTR-mediated FLuc activity did not result from aberrantly spliced mRNAs. The same result was obtained in A549 and HeLa cells expressing the pR_mTOR_F construct (Figure IV.3B).

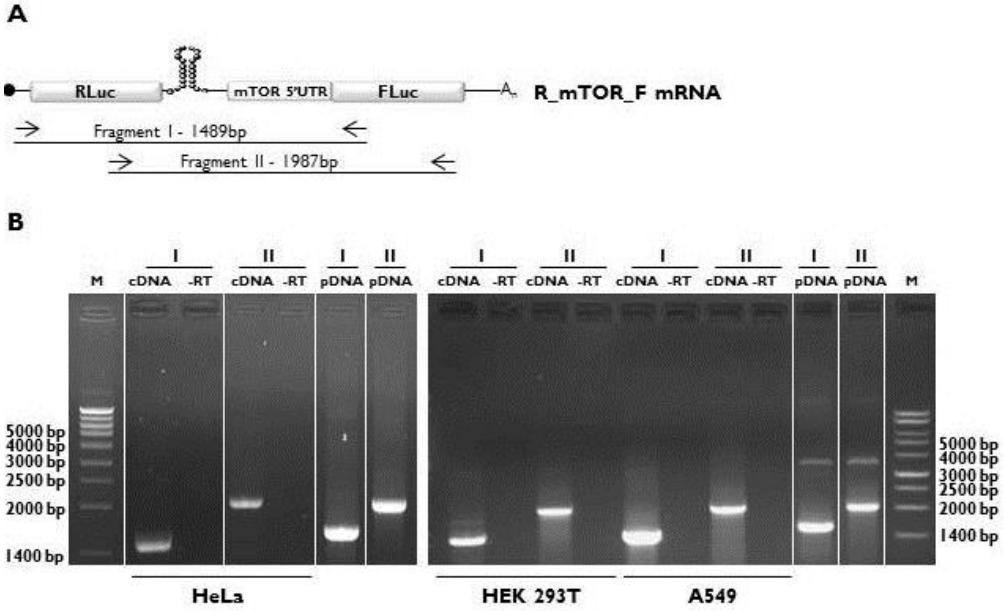


Figure IV.3. Dicistronic plasmid carrying human *MTOR* 5'UTR does not express alternatively spliced mRNAs. (A) Scheme depicting the dicistronic reporter R_mTOR_F mRNA expressed in HEK293T, HeLa and A549 cells. After transient transfection of the mentioned cell lines with the dicistronic pR_mTOR_F plasmid, RNA was extracted and the integrity of R_mTOR_F mRNA was analyzed by RT-PCR, which was performed with 2 pairs of primers (represented by arrows) originating overlapping fragments (I and II) spanning the entire transcript until the 3' end of Firefly luciferase (FLuc) cistron. **(B)** Ethidium bromide-stained agarose gel showing RT-PCR products. The amplified fragments, in the presence (cDNA) or in the absence of cDNA (-RT) are identified above the respective lane. The molecular weight marker (M) used is the NZY Ladder III. The correct full-length 1489bp fragment I was amplified from cDNA, while a longer fragment was amplified from plasmid DNA (pDNA), as it carries a 133bp chimeric intron upstream of the Renilla luciferase (RLuc) cistron. Amplification of fragment II revealed a 1987bp fragment in the cDNA samples and corresponding pDNA.

Potential cryptic promoter activity of *MTOR* 5'UTR was assessed by evaluating luciferase activity in promoterless reporters. SV40 promoter was removed from the dicistronic DNA constructs pR_F and pR_mTOR_F (Figure IV.4A; constructs 4 and 5) which were subsequently transfected into HEK293T, HeLa and A549 cells. As the

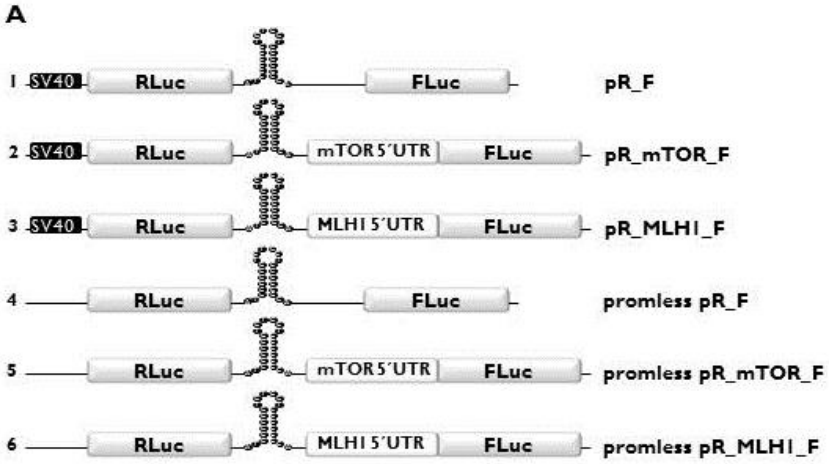
5'UTR of the human gene encoding the DNA mismatch repair MLH1 protein (NM_000249.3) contains a cryptic promoter (Arita et al., 2003; Ito et al., 1999), it was used as a positive control for cryptic promoter activity (Figure IV.4A; constructs 3 and 6). After transient co-transfection of each one of these constructs with a plasmid encoding β-galactosidase (β-gal) into HEK293T, HeLa, and A549 cells, Renilla and Firefly luciferase activities were determined and normalized to the activity of β-gal. The subsequent ratios of RLuc to β-gal or FLuc to β-gal (R/β-gal or F/β-gal) were compared to those from the empty pR_F vector (Figure IV.4B-D), arbitrarily set to 1. Results showed that in all the tested cell lines, removal of SV40 promoter decreased RLuc activity from all the constructs to background levels (Figure IV.4B-D).

In HeLa and A549 cells, a strong decrease in FLuc activity from the pR_F and pR_mTOR_F constructs was observed when the promoter was deleted, meaning that *MTOR* 5'UTR does not have cryptic promoter in these cells. On the other hand, the high levels of FLuc activity expressed from the dicistronic plasmid containing *MLH1* 5'UTR was unaffected by SV40 removal, as expected since it has a cryptic promoter (Figure IV.4C-D). A similar result was obtained from the pR_MLH1_F construct expressed in HEK293T cells; indeed, *MLH1* 5'UTR mediated FLuc production 19-fold comparing to that from pR_F empty construct, which remained unchanged upon removal of SV40 promoter (Figure IV.4B). In addition, in HEK293T cells, deletion of SV40 promoter induced a reduction of FLuc activity from pR_F and pR_mTOR_F constructs, which was less sharp than the reduction of RLuc activity, although significant (Figure IV.4B). Of note, the relative decrease in FLuc activity from the pR_mTOR_F promoterless construct comparing to its promoter-containing counterpart was similar to that observed for the empty construct (Figure IV.4B). FLuc coding region displays cryptic promoter activity (Vopalensky et al., 2008) and the fact

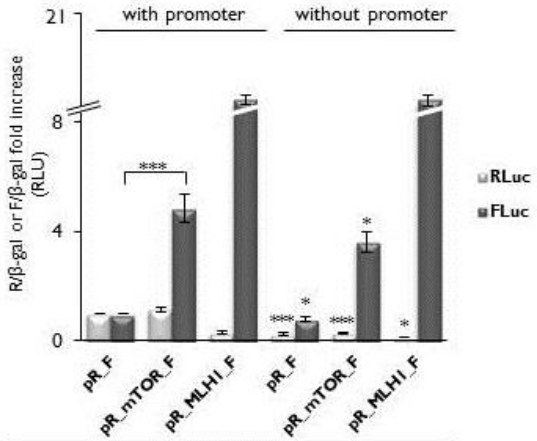
that its activity was reduced by removal of SV40 from the pR_F construct (Figure IV.4B) denotes that some translation reinitiation or ribosome read-through was occurring in this system. Though, the similar reduction of FLuc activity from the promoterless pR_F and pR_mTOR_F constructs in comparison with the SV40-containing counterparts demonstrated that those non-IRES events occur similarly in both constructs. Thus, these data point out that *MTOR* 5'UTR does not seem to display cryptic promoter activity.

IV.1.4. Human *MTOR* 5'UTR IRES activity does not require nuclear experience in HEK293T cells

One way to eliminate any contribution from cryptic promoters or splicing activation is to transfect cells with mRNA instead of plasmid DNA. Thus, to definitively confirm that *MTOR* 5'UTR-mediated FLuc activity is a result of cap-independent translation, transfection of dicistronic mRNAs was performed. For that, mRNAs were *in vitro* transcribed from the T7 RNA polymerase promoter-containing pR_F and pR_mTOR_F DNA reporters, capped, and polyadenylated. The integrity of the resultant mRNAs was confirmed on a formaldehyde-agarose gel (Figure IV.5A and B). Both mRNAs were transfected into HEK293T cells and the resulting RLuc and FLuc activities were measured (Figure IV.5C). The ratio of FLuc to RLuc (F/R) produced from R_mTOR_F mRNA was compared to that from the negative control, R_F mRNA, arbitrarily set to 1. Insertion of the *MTOR* 5'UTR segment resulted in a 2.2-fold increase in relative FLuc over the negative control (Figure IV.5C). These data unequivocally show that *MTOR* 5'UTR-mediated FLuc production is not due to cryptic promoter or splicing activation; instead, human *MTOR* 5'UTR shows IRES activity, which mediates cap-independent translation.

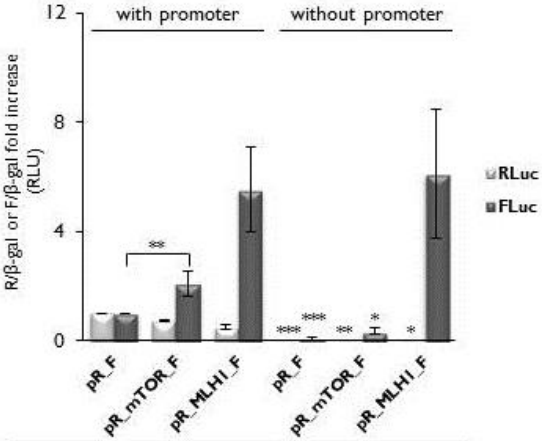


B. HEK293T cells



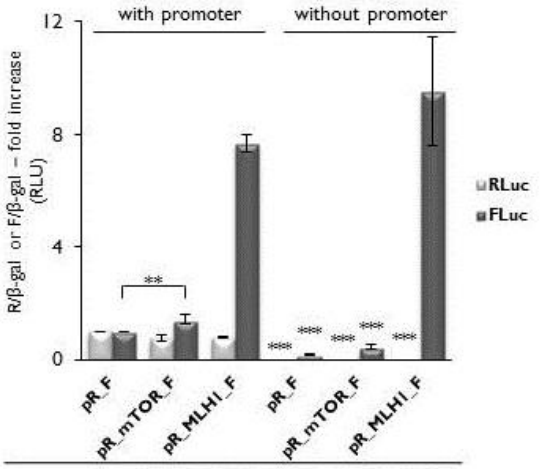
R/β-gal	1	1.15 ±0.08	0.31 ±0.07	0.26 ±0.05	0.29 ±0.04	0.12 ±0.006
F/β-gal	1	4.88 ±0.51	18.76 ±2.41	0.79 ±0.11	3.62 ±0.38	18.46 ±2.84

C. HeLa cells



R/β-gal	1	0.734 ±0.053	0.496 ±0.070	0.004 ±0.002	0.003 ±0.001	0.004 ±0.001
F/β-gal	1	2.089 ±0.475	5.533 ±1.549	0.082 ±0.043	0.356 ±0.132	6.107 ±2.351

D. A549 cells



R/β-gal	1	0.776 ±0.105	0.796 ±0.033	0.003 ±0.001	0.003 ±0.001	0.003 ±0.001
F/β-gal	1	1.436 ±0.167	7.669 ±0.299	0.183 ±0.012	0.461 ±0.078	9.533 ±1.937

Figure IV.4. *MTOR* 5'UTR does not display cryptic promoter. (A) Representation of the dicistronic reporter pR_F, pR_mTOR_F, and pR_MLH1_F constructs with and without SV40 promoter, as in Figure IV.2C. *MLH1* 5'UTR was used as a positive control for cryptic promoter activity. HEK293 **(B)**, HeLa **(C)** and A549 **(D)** cells were transiently co-transfected with each of the constructs described in (A), along with a plasmid expressing β-galactosidase (β-gal). Luciferase activity was measured 16 hours (HEK293T) or 24 hours (HeLa and A549) post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between Renilla luciferase (RLuc) and β-gal or Firefly luciferase (FLuc) and β-gal compared to that of the corresponding empty construct, which was arbitrarily set to 1. Data are presented below each graph as the means ± SD of three independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$; (∗∗) $p<0.01$; (∗∗∗) $p<0.001$.

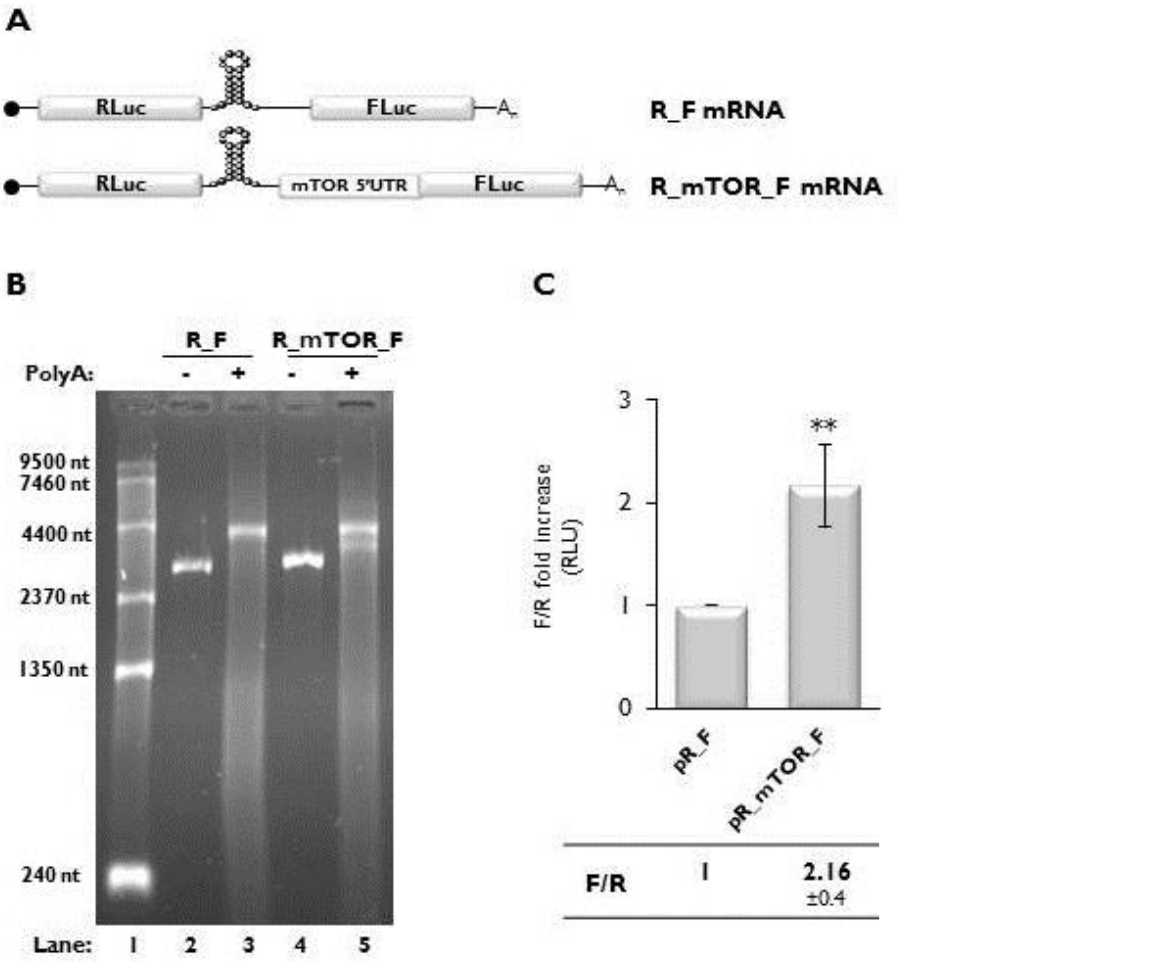


Figure IV.5. *In vitro* transcribed dicistronic mRNAs containing *MTOR* 5'UTR undergo IRES-driven translation after transient transfection. (A) Schematic illustration of the *in vitro* transcribed, capped and polyadenylated dicistronic R_F and R_mTOR_F reporter mRNAs. **(B)** Ethidium bromide-stained formaldehyde-agarose gel showing the integrity of 3µg of each mRNA before (-) and after (+) polyadenylation (polyA), as indicated. Lane 1 contains the 0.24-9.5kb RNA ladder (Invitrogen). **(C)** The capped and polyadenylated dicistronic mRNAs were transiently transfected into HEK293T cells and the Renilla (RLuc) and Firefly luciferase (FLuc) enzymatic activities

were measured 4 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between FLuc and RLuc, normalized to that of the R_F mRNA, which was arbitrarily set to 1. Data are presented below the graph as the means ± SD of four independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗∗) $p<0.01$.

IV.1.5. Hypoxic conditions with associated EIF2α phosphorylation and cap-dependent translation reduction stimulate *MTOR* IRES activity

The cellular response to hypoxia is characterized by HIF1α stabilization, that elicits a gene expression reconfiguration program (Manalo et al., 2005; Wang et al., 1995), characterized both by transcriptional induction of several genes and a translational reprogramming (Manalo et al., 2005; Arsham, 2003; Koritzinsky et al., 2006; Koumenis et al., 2002; Liu et al., 2006). Inactivation of EIF2B through phosphorylation of EIF2α, is one way by which, in hypoxia, protein synthesis is globally inhibited whereas a subset of mRNAs are preferentially translated, namely those encoding proteins involved in the hypoxic response (Koritzinsky and Wouters, 2007; Koritzinsky et al., 2006, 2007; Koumenis et al., 2002). Since MTORC1 is involved in the hypoxic response by regulating expression of HIF1α and modulating its transcriptional activity (Bernardi et al., 2006; Hudson et al., 2002; Land and Tee, 2007; Laughner et al., 2001; Thomas et al., 2006; Zhong et al., 2000), it was hypothesized that *MTOR* IRES-driven translation might be affected by hypoxic conditions. To test this, HEK293T cells were transiently transfected with the pR_F, pR_HBB_F, pR_mTOR_F, or pR_c-myc_F constructs 2 hours before treatment with the hypoxia-mimicking agent cobalt chloride ($CoCl_2$) (200μM) for 20 hours. The cellular hypoxic stimulus was monitored by Western blot with an anti-HIF1α antibody; and α-tubulin was used as a loading control. As seen in Figure IV.6A, 200μM of $CoCl_2$ stabilized the O_2-sensitive HIF1α subunit and increased the phosphorylated EIF2α (P-EIF2α) protein levels. Total EIF2α levels, on the contrary, were not considerably affected by this treatment (Figure IV.6A; lower panel).

Enhanced EIF2α phosphorylation may be considered as an indicator for reduction in global cap-dependent translation. Nevertheless, to better measure the effect of hypoxia on cap-dependent translation, the *MYC* IRES-containing dual luciferase reporter was used to distinguish cap-dependent *versus* cap-independent translation, as it is known that *MYC* IRES is unresponsive to hypoxic stress (Lang et al., 2002). Thus, the RLuc/FLuc ratio determines the cap-dependent translation status in the hypoxic-mimicking conditions used. As seen in Figure IV.6B, hypoxia attenuated cap-dependent translation by about 35% when compared to the control condition. In these settings, the endogenous MTOR protein levels were also monitored revealing that, although cap-dependent translation was compromised, MTOR protein levels remained unchanged (Figure IV.6A,B). To test whether the maintenance of MTOR levels is due to increased cap-independent *MTOR* IRES activity, FLuc/RLuc ratios expressed from the pR_F, pR_HBB_F, and pR_mTOR_F constructs, under normoxic and hypoxic conditions, were also evaluated by dual luciferase assays and relative luciferase activity of each construct was compared to that obtained from the empty pR_F construct at the corresponding condition (Figure IV.6C). It is observed that, in fact, relative *MTOR* IRES activity was significantly potentiated under hypoxia. As expected, the activity from the negative control remained unaltered (Figure IV.6C).

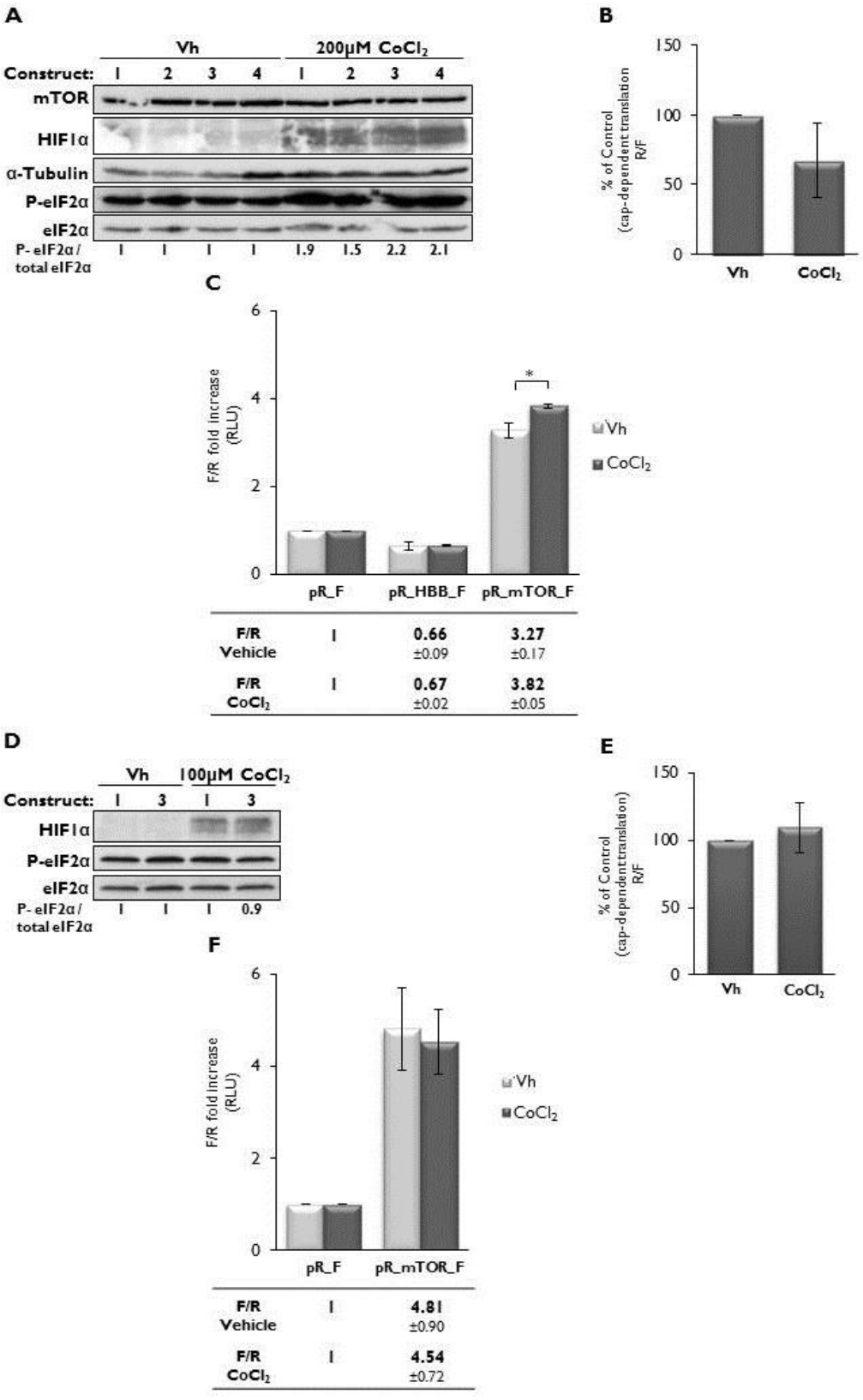


Figure IV.6. Relative *MTOR* IRES activity is enhanced in $CoCl_2$-induced hypoxia when EIF2α is phosphorylated. HEK293T cells were transiently transfected with the dicistronic constructs pR_F, pR_HBB_F, pR_mTOR_F and pR_c-myc_F (Figure IV.2C) and treated 2 hours later with drug vehicle (Vh), 200µM-$CoCl_2$

(A-C), or 100µM-$CoCl_2$ (D-F) for 24 hours. **(A)** Phosphorylation of EIF2α by 200µM-$CoCl_2$ has no impact on endogenous MTOR protein levels. Western blot analysis of transfected HEK293T cell extracts treated with 200µM-$CoCl_2$, using antibodies against MTOR, HIF1α, total and phosphorylated EIF2α (P-EIF2α) proteins. The α-tubulin specific antibody controls for protein loading. HIF1α stabilization demonstrates hypoxia induction and EIF2α phosphorylation is demonstrated by increased P-EIF2α/EIF2α ratio. **(B)** Cap-dependent translation is reduced in 200µM-$CoCl_2$ treated cells. *In vivo* cap-dependent translational assays were performed using the dual Renilla/Firefly luciferase (R/F) assay, in extracts from HEK293T transfected with pR_c-myc_F plasmid and treated with 200µM-$CoCl_2$. The values are shown as the luminescence (RLuc/FLuc) ratio, normalized to that of the pR_c-myc_F construct in control conditions (Vh), which was arbitrarily set to 100. **(C)** Hypoxia with associated EIF2α phosphorylation and cap-dependent translation reduction increases relative *MTOR* IRES activity. Luminescence assays were performed using extracts from 200µM-$CoCl_2$-treated cells described in (A). The values (relative light units; RLU) are shown as the luminescence (FLuc/RLuc) ratio, normalized to that of the pR_F construct in each condition, arbitrarily set to 1. Data are presented as the means ± SD from three independent experiments. Statistical analysis was performed using Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$. **(D)** Cellular treatment with 100µM-$CoCl_2$ induces hypoxia with no EIF2α phosphorylation. Western blot analysis of extracts from HEK293T cells transfected with pR_F and pR_mTOR_F plasmids and treated with 100µM-$CoCl_2$. HIF1α stabilization demonstrates hypoxia induction but with unchanged P-EIF2α/EIF2α ratio. **(E)** In hypoxic conditions without EIF2α phosphorylation, cap-dependent translation is not inhibited. *In vivo* cap-dependent translational assays were performed in extracts from 100µM-$CoCl_2$-treated cells as in B. **(F)** Chemical hypoxia with no EIF2α phosphorylation does not affect relative *MTOR* IRES activity. Extracts from 100µM-$CoCl_2$-treated HEK293T cells transfected with pR_F and pR_mTOR_F plasmids were used to measure luciferase activity by luminometry assays as in C.

Furthermore, the observed increase of relative *MTOR* IRES activity was not attributed to HIF1α induction *per se* since, in hypoxic conditions that induced HIF1α expression but had no impact on EIF2α phosphorylation nor on cap-dependent translation – 100µM $CoCl_2$ cellular treatment – (Figure IV.6D and E), there was no stimulation of *MTOR* IRES activity (Figure IV.6F). Thus, the results illustrate that hypoxic conditions with associated EIF2α phosphorylation increase relative *MTOR* IRES activity, in a HIF1α induction-independent manner.

IV.1.6. *MTOR* IRES-driven translation is stimulated by ER stress both in the anti- and pro-apoptotic unfolded protein response output, with a more pronounced effect when EIF2α phosphorylation is increased

UPR is activated when ER integrity is disrupted by accumulation of unfolded or misfolded proteins, alterations in calcium stores, disturbances of the redox balance in the ER lumen and oxidative stress (reviewed in Ron and Walter, 2007). PERK activation, that triggers one of the UPR branches, increases EIF2α phosphorylation which reduces the influx of proteins into the ER and induces a translational reprogramming (Harding et al.,, 1999; Harding et al., 2000a). As UPR and MTORC1 signalling mutually regulate each other (reviewed in Appenzeller-Herzog and Hall, 2012), it was hypothesized that *MTOR* is one of the transcripts preferentially translated upon ER stress-induced UPR. For that, HEK293T cells were transiently transfected with the dicistronic DNA plasmids pR_F, pR_HBB_F, pR_mTOR_F and pR_c-myc_F (Figure IV.2C) and treated with 1μM Thapsigargin (TG) for 20 hours. TG induces ER stress by reducing ER Ca^{2+} stores, leading to an increase of EIF2α phosphorylation and global translation inhibition (Thastrup et al., 1990; Wong et al., 1993). Figure IV.7A shows that TG treatment resulted in increased EIF2α phosphorylation as demonstrated by the augmented ratio between P-EIF2α and total EIF2α protein levels (P-EIF2α/EIF2α) in TG-treated *versus* untreated cells (Figure IV.7A). Knowing that the primary homeostatic and anti-apoptotic outcome of UPR is reversed by prolonged or severe ER stress, in which cells undergo apoptosis (reviewed in Tabas and Ron, 2011), the apoptotic output of the used experimental conditions was analyzed. The molecular marker used to test apoptosis was cleavage of the nuclear DNA repair enzyme poly (ADP-ribose) polymerase (PARP), which suffers proteolytic cleavage in response to

many apoptotic stimuli (Oliver et al., 1998). It is observed that PARP cleavage increases in TG-treated cells comparing to DMSO Vh-treated control cells (Figure IV.7A). Thus, increased EIF2α phosphorylation paralleled the apoptotic output of UPR, which is in agreement with its pro-apoptotic role (Allagnat et al., 2011; McCullough et al., 2001). To quantify the effect of TG treatment on cap-dependent translation, the same strategy as before for the hypoxic conditions was used. As seen in Figure IV.7B, TG treatment attenuated cap-dependent translation by about 40% (Figure IV.7B). Under these conditions, it is also noticed that MTOR protein levels remained unchanged relatively to the control conditions (Figure IV.7A). It was then tested whether this maintenance of MTOR protein levels were due to increased cap-independent translation of *MTOR* mRNA. For that, FLuc/RLuc ratios expressed from the pR_F, pR_HBB_F and pR_mTOR_F constructs (Figure IV.2C) in cells under DMSO or TG treatment, were also evaluated by dual luciferase assays as above. It is observed that relative *MTOR* IRES activity was significantly increased from 3.45-fold to 6.81-fold in DMSO- *versus* TG-treated cells, respectively (Figure IV.7C). Together, these data show that IRES-driven translation of *MTOR* mRNA is stimulated by the ER stress-induced pro-apoptotic UPR.

Next, it was investigated whether *MTOR* IRES is also stimulated by the anti-apoptotic phase of UPR. For that, a set of the above indicated transfected cells was treated with low concentrations of TG (0.25μM) or Vh (DMSO), tested for PARP cleavage and EIF2α phosphorylation as before. As demonstrated in Figure IV.7D, no increase of EIF2α phosphorylation and no cleaved PARP were observed. Instead, some cleaved PARP was detected at DMSO-treated cells, which may reflect the confluence of control cultured cells (Figure IV.7D). These results indicate that these conditions of

TG treatment triggered the anti-apoptotic phase of UPR. In this stage, the endogenous levels of MTOR protein were also maintained (Figure IV.7D).

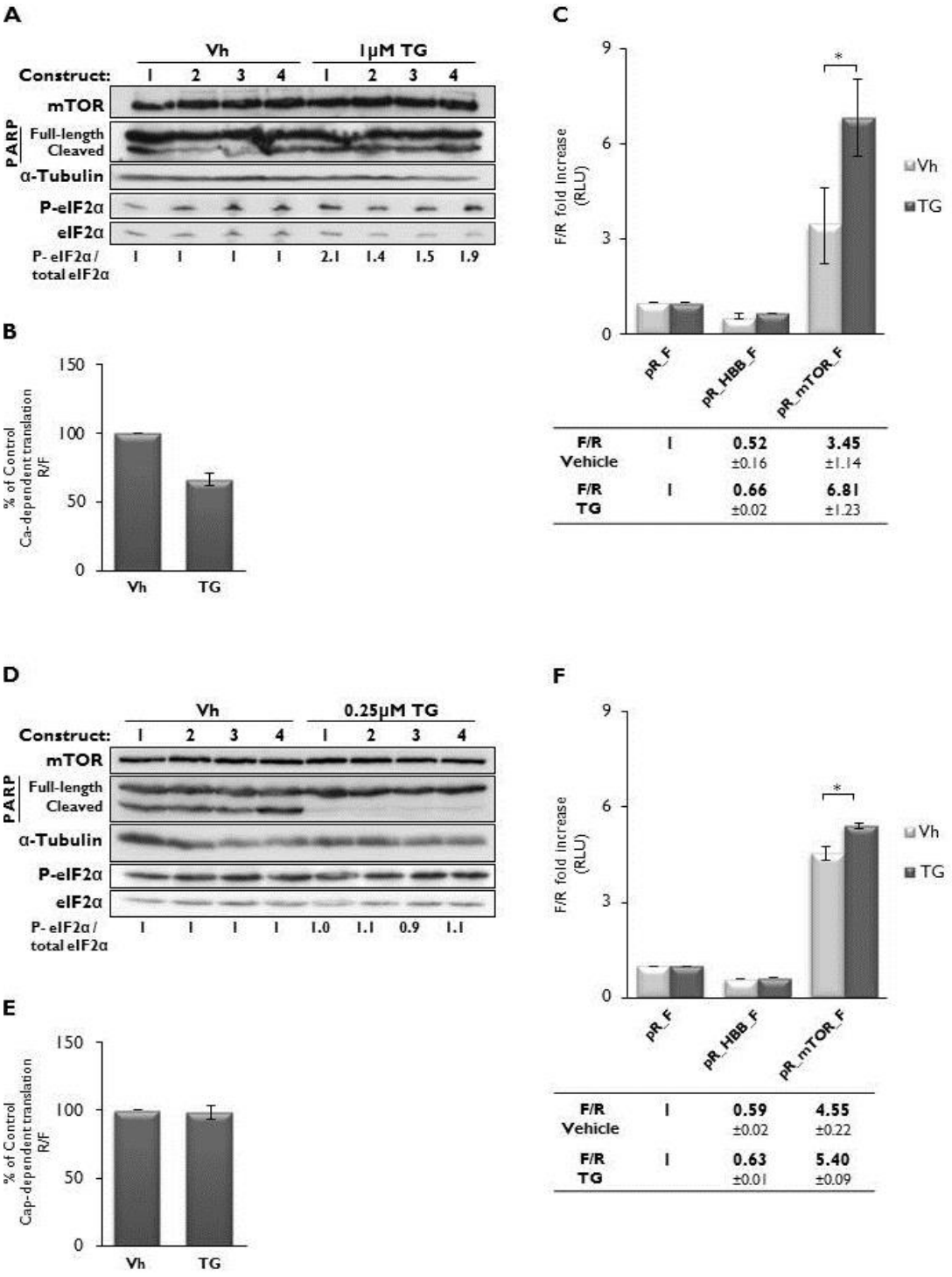


Figure IV.7. Relative *MTOR* IRES activity is enhanced by the unfolded protein response (UPR). HEK293T cells were transiently transfected with the dicistronic constructs pR_F, pR_HBB_F, pR_mTOR_F and pR_c-myc_F (Figure IV.2C) and treated 19 hours later with DMSO (Vh), 1μM-thapsigargin (TG) (A-C), or

0.25µM-TG (D-F) for 20 hours. **(A)** Phosphorylation of EIF2α by 1µM-TG treatment has no impact on endogenous MTOR protein levels. Western blot analysis of extracts from transfected and 1µM-TG treated HEK293T cells, using antibodies against MTOR, PARP, total and phosphorylated EIF2α (P-EIF2α) proteins. The α-tubulin specific antibody controls for protein loading. PARP cleavage demonstrates induction of pro-apoptotic phase of UPR and EIF2α phosphorylation is demonstrated by increased P-EIF2α/EIF2α ratio. **(B)** Cap-dependent translation decreases in 1µM-TG treated cells. *In vivo* cap-dependent translational assays were performed using the dual Renilla/Firefly luciferase (R/F) assay, in extracts from 1µM-TG treated HEK293T cells transfected with pR_c-myc_F plasmid. The values are shown as the luminescence (RLuc/FLuc) ratio, normalized to that of the pR_c-myc_F construct in control conditions (Vh), which was arbitrarily set to 100. **(C)** Pro-apoptotic UPR with EIF2α phosphorylation increases relative *MTOR* IRES activity. Luminescence assays were performed using extracts from 1µM-TG-treated cells described in (A). The values (relative light units; RLU) are shown as the luminescence FLuc/RLuc ratio, normalized to that of the pR_F construct in each condition, arbitrarily set to 1. Data are presented as the means ± SD from three independent experiments. Statistical analysis was performed using Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$. **(D)** Treatment with 0.25µM-TG induces the anti-apoptotic UPR with unphosphorylated EIF2α. Western blot analysis of extracts from transfected and 0.25µM-TG treated HEK293T cells. Reversal of PARP cleavage demonstrates the anti-apoptotic output of UPR, in which the EIF2α/EIF2α ratio is unchanged. **(E)** In the anti-apoptotic UPR with unphosphorylated EIF2α, cap-dependent translation is not inhibited. *In vivo* cap-dependent translational assays were performed in extracts from 0.25µM-TG-treated cells as in B. **(F)** The anti-apoptotic UPR with unphosphorylated EIF2α stimulates relative *MTOR* IRES activity. Extracts from 0.25µM-TG-treated HEK293T cells transfected with pR_F, pR_HBB_F and pR_mTOR_F constructs were used to measure luciferase activity by luminometry assays as in C.

The effect of the anti-apoptotic outcome of ER stress on cap-dependent translation was quantified as above. The results indicated that cap-dependent translation is not affected by treatment with 0.25µM TG, which is in accordance with the absence of increased EIF2α phosphorylation (Figure IV.7D and E). Analysis of *MTOR* IRES activity was performed as previously described and demonstrated that, in the anti-apoptotic phase of UPR, relative *MTOR* IRES activity increased from 4.55-fold to 5.40-fold over the background (Figure IV.7F). However, comparing to the results obtained when cells are in the pro-apoptotic stage of UPR (Figure IV.7C *versus* IV.4F), the increase in *MTOR* IRES activity was less accentuated. Together these data show that relative *MTOR* IRES activity is enhanced by the anti-apoptotic outcome of UPR with

unphosphorylated EIF2α, but a more robust effect is observed in the pro-apoptotic phase with increased EIF2α phosphorylation.

IV.1.7. *MTOR* IRES activity is stimulated by rapamycin-induced MTORC1 inactivation

A major function of MTORC1 is to regulate protein synthesis through phosphorylation of several substrates including S6Ks and the inhibitory 4EBPs (Blommaart et al., 1995; Hara et al., 1998). Based on these data, it was examined whether *MTOR* IRES-mediated translation is affected upon MTORC1 signaling inactivation by rapamycin treatment. For that, HEK293T cells were transiently transfected with the dicistronic DNA plasmids pR_F, pR_HBB_F, pR_mTOR_F and pR_c-myc_F plasmids (Figure IV.2C) and treated 14 hours later with 80nM rapamycin for no more than 6 hours, to prevent the confounding effects of MTORC2 inactivation as well (Sarbassov et al., 2006). In parallel, a similar set of control transfected cells was treated with vehicle (DMSO). MTORC1 inhibition was evaluated by the phosphorylation status of the downstream S6K1 effector. As seen in Figure IV.8A, rapamycin blocked canonical MTORC1-dependent phosphorylation of S6K1 and, as expected, did not increase phosphorylation of EIF2α (Figure IV.8A; lower panels). As *MYC* translation is unchanged upon MTORC1 inactivation (Thoreen et al., 2012), the *MYC* IRES-containing dicistronic construct was used to quantify how much cap-dependent translation was compromised in these conditions. Results showed that cap-dependent translation efficiency was affected to some extent (Figure IV.8B), which is in accordance with previous results reporting that rapamycin only partially reduces cap-dependent translation (Choo et al., 2008). In these conditions, the dicistronic mRNAs translation was examined to assess *MTOR* IRES-mediated translation in rapamycin- *versus* DMSO-

treated cells. The obtained results showed that rapamycin exposure significantly stimulated relative *MTOR* IRES activity (Figure IV.8C). As expected, the activity from the negative control remained unchanged (Figure IV.8C). Together these data demonstrate that relative *MTOR* IRES activity is stimulated by rapamycin-induced MTORC1 inactivation.

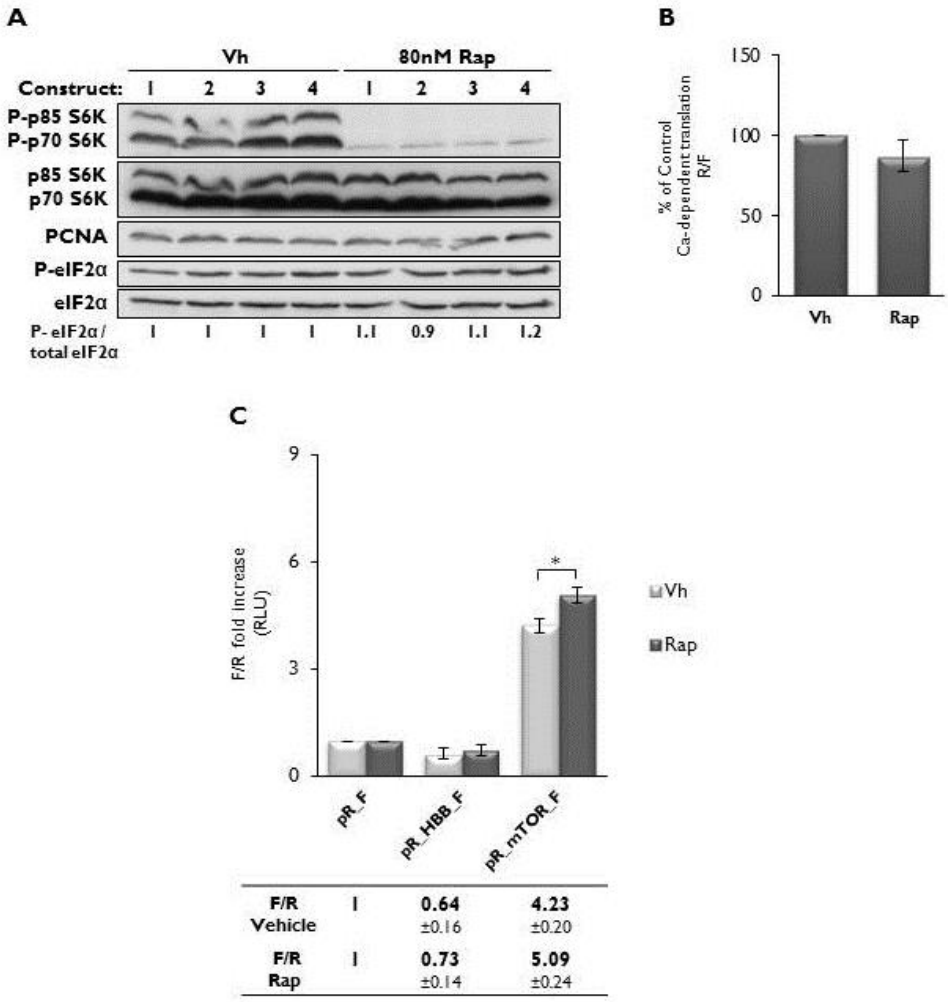


Figure IV.8. Relative *MTOR* IRES activity is stimulated by rapamycin-induced MTORC1 inactivation. HEK293T cells were transiently transfected with the dicistronic constructs pR_F, pR_HBB_F, pR_mTOR_F and pR_c-myc_F (Figure IV.2C) and treated 14 hours later with 80nM-rapamycin (Rap) or equal volume of DMSO (Vh) for 6 hours. **(A)** MTORC1 inactivation by Rap treatment. Western blot analysis of transfected HEK293T cell extracts treated with 80nM-Rap, as indicated, using antibodies against total and phosphorylated p85 and p70 (P-p85 and P-p70) S6K proteins, as well as total and phosphorylated EIF2α (P-EIF2α) proteins. The proliferating cell nuclear antigen (PCNA) specific antibody controls for protein loading. Inhibition of phosphorylation of p85 and p70 S6Ks demonstrates MTORC1

inactivation, and unphosphorylation of EIF2α is demonstrated by unchanged levels of total EIF2α and P-EIF2α proteins. **(B)** In conditions of MTORC1 inactivation and unphosphorylated EIF2α, cap-dependent translation decreases. *In vivo* cap-dependent translational assays were performed using the dual Renilla/Firefly luciferase (R/F) assay, in extracts from 80nM-Rap-treated HEK293T cells transfected with pR_c-myc_F plasmid. The values are shown as the luminescence (RLuc/FLuc) ratio, normalized to that of the pR_c-myc_F construct in control conditions (Vh), which was arbitrarily set to 100. **(C)** Inactivation of MTORC1 with no EIF2α phosphorylation, increases relative *MTOR* IRES activity. Luminescence assays were performed using extracts from 80nM-Rap-treated cells described in (A). The values (relative light units; RLU) are shown as the luminescence ratio between FLuc and RLuc, normalized to that of the pR_F construct in each condition, arbitrarily set to 1. Data are presented as the means ± SD from three independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$.

IV.2. Colorectal cell malignization and metastization is accompanied by differential increase in translation driven by different IRESs elements

Deregulation of cap-dependent mRNA translation is critical for induction of colorectal cell transformation and metastization (She et al., 2010; Ye et al., 2013). Given the role of IRES-mediated translation on tumorigenesis, as previously mentioned, we wanted to test whether IRES-dependent translation varies between different colorectal cancer (CRC) stages. For that, the IRES-mediated translation driven by *MTOR*, *MYC* and EMCV IRESs in NCM460 cells (derived from normal intestinal mucosa), Sw480 cells (derived from a moderately differentiated adenocarcinoma of descending colon) and in the metastatic HCT116 cell line (derived from poorly differentiated colon cancer) was assessed. Those cell lines were transiently transfected with the dicistronic pR_F, pR_HBB_F, pR_mTOR_F, pR_c-myc_F and pR_EMCV_F (Figure IV.2C) and the F/R ratios were obtained as previously. Results demonstrated that the three IRESs are active in all the tested cell lines though at different degrees (Figure IV.9). The NCM460 cell line presents the lowest IRES activation level of the three IRESs, paralleling the levels observed in HCT116 cells for *MYC* and *MTOR*. Both *MYC* and *MTOR* IRESs are more activate in the Sw480 cell line. On the other hand, EMCV IRES

is equally activated in NCM460 and Sw480 cells and is highly potentiated in HCT116 cells (Figure IV.9). Together our results suggest that different IRES elements respond differently to colorectal cell malignant transformation and metastization. Whereas the activation level of the cellular *MYC* and *MTOR* IRESs seems to increase upon cellular malignization, a phenomenon that is reversed upon CRC metastization, the viral EMCV IRES presents the highest level at a metastatic CRC stage.

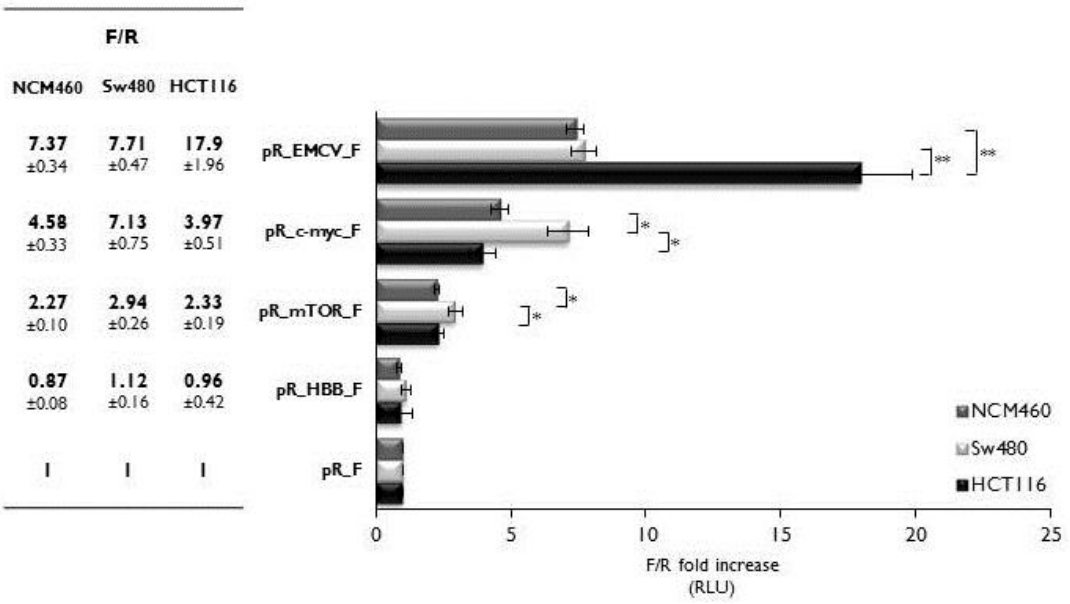


Figure IV.9. The enhancement of IRES-mediated translation of cellular mRNAs induced by colorectal cell malignant transformation is reversed by metastization whereas EMCV IRES activity is only enhanced in a metastatic stage. NCM460, Sw480 and HCT116 cell lines were transiently transfected with the dicistronic constructs pR_F, pR_HBB_F, pR_mTOR_F, pR_c-myc_F and pR_EMCV_F (Figure IV.2C). Luciferase activity was measured 24 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between Firefly luciferase (FLuc) and Renilla luciferase (RLuc), normalized to that of the empty construct, arbitrarily set to 1. Data are means ± standard deviation (SD) of at least 3 independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$; (∗∗) $p<0.01$.

IV.3. IRES-dependent synthesis of Δ160P53

IV.3.1. Expression of Δ160P53 isoform is translationally regulated upon stress conditions

The *Δ133P53* transcript, originated from the *TP53* intragenic promoter, generates the Δ133P53 isoform and a 32 kDa-protein isoform, referred as Δ160P53. Given that this protein isoform results from mRNA translation initiation at codon 160 (Marcel et al., 2010b), it was hypothesized that it is regulated by an IRES element. To assess that, first, the expression levels of Δ160P53 protein isoform in stress conditions were evaluated. For that, cell lines expressing endogenous P53 protein (human lung carcinoma A549 and HCT116 cells) as well as H1299, a human lung carcinoma P53-null cell line transiently transfected with a plasmid containing the cDNA for the *Δ133P53* transcript (Δ133); were treated with the ER stress-inducer TG (Thastrup et al., 1990; Wong et al., 1993). Full length and P53 isoforms were detected by Western Blot with P53-specific CM1 antibody, and α-tubulin was used as a loading control. As seen in Figure IV.10A, the hardly detectable endogenous levels of Δ160P53 protein in normal conditions were increased upon ER stress, in A549 and HCT116 cells. Furthermore, Δ160P53 protein expression from the *Δ133P53* cDNA-containing plasmid was induced upon TG-triggered ER stress, in H1299 cells (Figure IV.10A). In order to test whether the induction of Δ160P53 was limited to ER stress A549, mouse embryonic fibroblast NIH3T3 and Δ133-transfected H1299 cell lines were subjected to another type of stimulus, cell confluency above 100% (over-confluency or O-C), and Δ160P53 protein levels were detected as previously mentioned. Figure IV.10B shows that the low endogenous levels of Δ160P53 protein observed in unstressed cells are increased by stress triggered by over-confluency, in A549 cells. Similarly, Δ160P53 protein

expression from the *Δ133P53* cDNA-containing plasmid is increased by O-C–induced stress, in H1299 cells. Unstressed NIH3T3 cells did not express neither Δ133P53 nor Δ160P53 protein isoforms, but over-confluency induces a ~32kDa-protein and comparison with the protein isoforms expressed in H1299 transfected with a plasmid carrying the cDNA for wild-type *Δ133P53* transcript or containing a substitution from methionine to alanine at codons 133 (M133A Δ133), revealed that this protein corresponds to the Δ160P53 isoform. Interestingly, in overconfluent NIH3T3 cells, the protein levels of Δ160P53 were even greater than those of P53 (Figure IV.10B). These data show that induction of Δ160P53 protein isoform is not exclusively triggered by ER stress, and extends to cellular over-confluency–triggered stress. Next, it was tested whether the induction of Δ160P53 resulted from increased protein production or decreased protein degradation. First, the P53-null cell line H1299 was transiently transfected with the aforementioned plasmid containing the cDNA for wild-type *Δ133P53* transcript or containing a substitution from methionine to alanine at codons 133 (M133A Δ133) or 160 (M160A Δ133), respectively; and the protein levels of each isoform was analyzed. Results showed that mutating the initiation start site at codon 160 abolished Δ160P53 expression whereas mutating AUG133 actually increased Δ160P53 levels (Figure IV.10C). The fact that Δ160P53 protein isoform is produced even when Δ133P53 is not expressed means that the observed induction of this protein results from post-transcriptional regulation. In order to check whether this event relies on augmented mRNA translation or protein stability, *Δ133P53*-transfected H1299 cells were treated with cyclohexamide (CHX), a translation elongation inhibitor (Obrig et al., 1971) or carbobenzoxy-Leu-Leu-leucinal (MG132), a proteasome inhibitor (reviewed in Lee and Goldberg, 1998), alone or in combination with tunicamycin (TU), a ER-stress inducer (Brandish et al., 1996; HEIFetz et al., 1979;

Keller et al., 1979). Results demonstrated that Δ160P53 protein was induced by TU treatment when protein degradation was impaired and that this induction was similar to the observed when cells were only treated with the ER stress-activating drug. On the other hand, CHX treatment reversed the TU-triggered Δ160P53 induction (Figure IV.10D). Together these data demonstrate that Δ160P53 expression is induced by cellular stress conditions through increased mRNA translation.

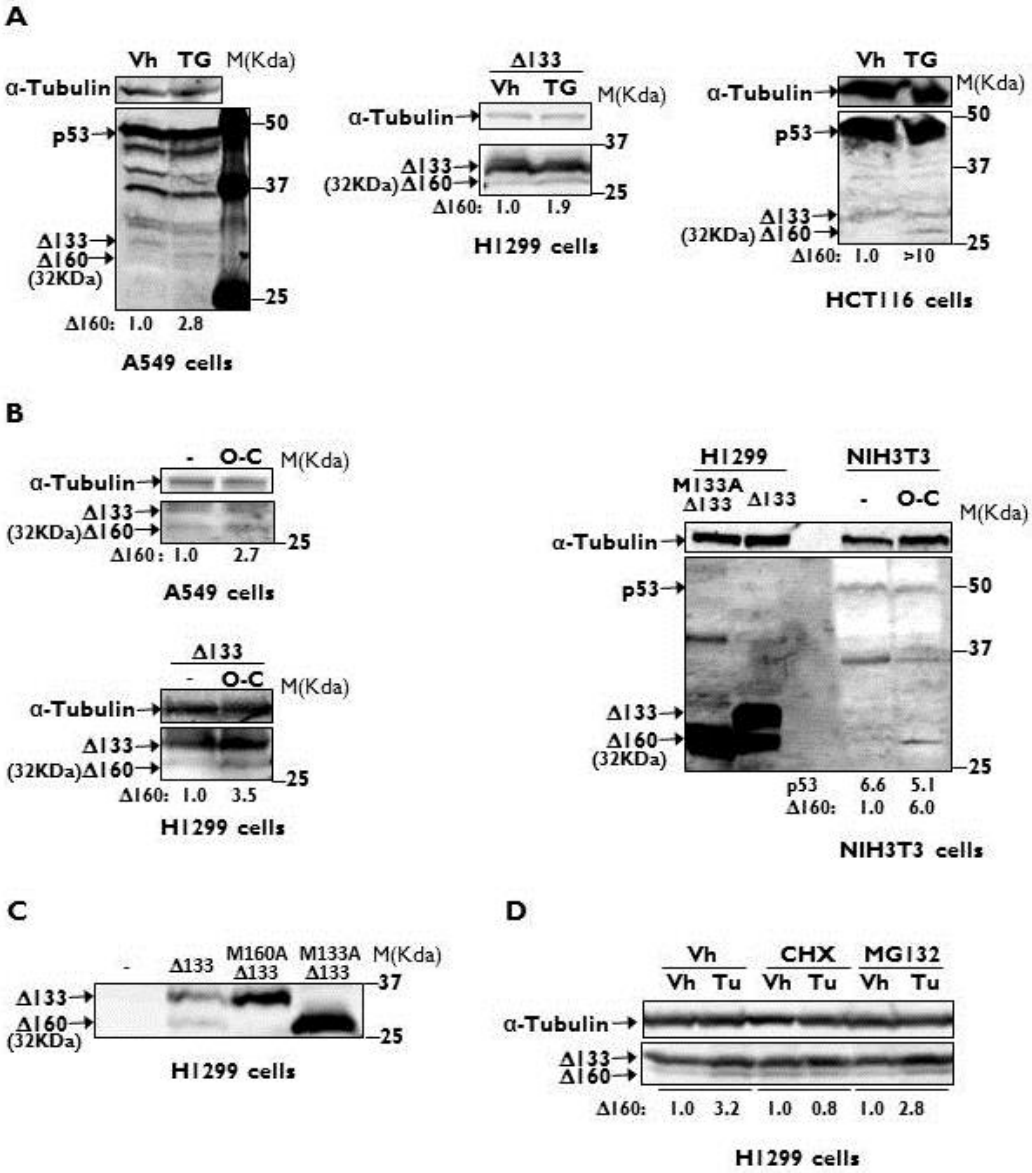


Figure IV.10. Δ160P53 protein isoform is induced upon stress conditions, through a translational regulatory mechanism. (A) Δ160P53 protein expression is induced by Endoplasmic Reticulum (ER) stress. A549 and HCT116 cells were treated with 1μM Thapsigargin (TG) or DMSO vehicle (Vh) for 20 hours; and H1299 cells were transiently transfected with a plasmid containing the cDNA for the

wild-type *Δ133P53* transcript (Δ133) for 19 hours and treated with 1μM Thapsigargin (TG) during 20 hours. Western blot analysis of extracts of A549, HCT116 and transfected H1299, using antibody CM-1 for detection of P53 and against human α-tubulin, for control of protein loading, is shown. Molecular weight markers (M) were used to confirm the identity of the isoforms. **(B)** Δ160P53 protein expression is induced by over-confluency (O-C). A549 and NIH3T3 untransfected cells, and H1299 cells transiently transfected with a plasmid containing the cDNA for the wild-type *Δ133P53* transcript, were grown during 2-10 days to achieve O-C or collected at a density of 300cells/mL (normal cell density). To confirm the identity of the isoforms, H1299 cells were transiently transfected with a plasmid containing the cDNA for the wild-type *Δ133P53* transcript or containing a substitution from methionine to alanine at codons 133 (M133A Δ133) (right panel). Western Blot analysis of the correspondent extracts, performed as in A., is shown. **(C)** A post-transcriptional event is responsible for Δ160P53 protein induction. H1299 cells were transiently transfected with a plasmid containing the cDNA for the wild-type *Δ133P53* transcript or containing a substitution from methionine to alanine at codons 133 (M133A Δ133) or 160 (M160A Δ133). Western Blot analysis of the correspondent extracts, performed as in A, is shown. **(D)** Δ160P53 protein induction is a result of increased protein synthesis. H1299 cells were transiently transfected with a plasmid containing the cDNA for the wild-type *Δ133P53* transcript (Δ133) for 24 hours and treated with 12μM Tunicamycin (TU) or DMSO vehicle (Vh), alone or in combination with 10μg/mL cyclohexamide (CHX) or 25μM MG132 for 2 hours. The correspondent extracts were analyzed by Western Blot as in A.

IV.3.2. Δ160P53 isoform is produced by cap-independent translation through a coding region located-IRES element, whose activity is stimulated by ER stress and is inhibited by its 5´UTR

The fact that Δ160P53 protein isoform is a result of internal initiation at codon 160 (Marcel et al., 2010b), in combination with the previous findings demonstrating that its expression increases through a translational event, in stress conditions in which overall protein synthesis is reduced (Figure IV.7; Wong et al., 1993), might indicate that an IRES element is driving Δ160P53 protein production. To assess that, a dicistronic DNA reporter containing enhanced green fluorescent protein (EGFP) ORF as the first cistron and Δ160P53 coding region as the second cistron was used. The same hairpin used in the previous described pR_F dicistronic reporter was cloned downstream of EGFP, originating the pE_Δ160_ORF construct. The region from -78-nt to Δ160P53

ATG was considered as its putative 5´UTR and was cloned upstream of Δ160P53 coding region, originating the pE_5´Δ160_ORF reporter (Figure IV.11A). H1299 cells were transfected with the abovementioned dicistronic constructs and treated with TG. Δ160P53 protein isoform expression was analyzed as previously. As seen in Figure IV.11B, Δ160P53 protein expressed either from the 5´UTR-containing or the 5´UTRless dicistronic plasmids was induced by TG treatment. Surprisingly, the protein levels of Δ160P53 expressed from the 5´UTRless construct were higher than that of Δ160P53 5´UTR-containing reporter plasmid (Figure IV.11B). These data indicate that Δ160P53 is cap-independent translated through an IRES element located downstream of AUG160 and that its 5´UTR is inhibitory.

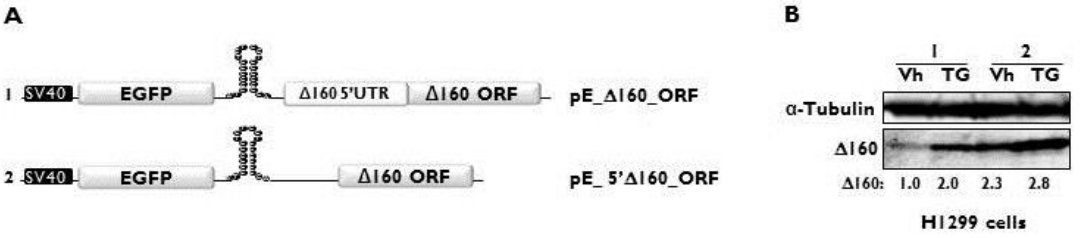


Figure IV.11. An IRES element within Δ160P53 coding region supports its translation, in a dicistronic reporter DNA construct with impaired reinitiation, and its putative 5´UTR is inhibitory. (A) Scheme depicting the dicistronic constructs pE_ Δ160_ORF and pE_ 5'Δ160_ORF. A stable hairpin was cloned downstream of the Enhanced Green Fluorescent Protein (EGFP) open reading frame (ORF) (EGFP box) and upstream of Δ160P53 coding region (Δ160P53 box) alone or preceded by its putative 5´UTR (nucleotide -78 to – 1, in which + 1 is the A from Δ160P53 ATG), to create pE_ Δ160_ORF and pE_ 5'Δ160_ORF constructs, respectively. The dicistronic transcriptional units are under the control of SV40 promoter. **(B)** Western blot analysis of extracts of H1299 cells transiently transfected with plasmids depicted in A and treated, 19 hours later, with 1μM Thapsigargin (TG) or DMSO (Vh) for 20 hours, using antibody CM1 for detection of P53 and α-tubulin for control of protein loading.

A more detailed study of this IRES element was performed using the previously described pR/F dicistronic DNA reporter. The *HBB* 5´UTR was used as a negative control for IRES activity (Lockard and Lane, 1978) and the positive control was the

MYC IRES (Stoneley et al., 1998), as previously. The DNA regions including Δ160P53 coding region until nt 432 alone or proceeded by its putative 5´UTR, as well as the Δ160P53 coding region until nucleotide 258 were cloned upstream of FLuc, originating pR_Δ160nt432_F, pR_ 5´Δ160nt432_F and pR_ Δ160nt258_F respectively (Figure IV.12A). H1299 and HCT116 cells were transiently transfected with the aforementioned plasmids, luciferase activity was measured and the ratio between FLuc and RLuc was compared to that of the empty constructs. The results showed that Δ160P53 coding region induces cap-independent production of FLuc in both cell lines, to a similar extent. Inclusion of the 5´UTR abolished IRES-mediated synthesis of Δ160P53 in both cell lines (Figure IV.12B). The minimal RNA length used that turned out positive for IRES activity was 432 nts from AUG160, as the Δ160P53 coding sequence until nt 258 was negative for IRES activity (Figure IV.12C).

In order to test whether Δ160P53 IRES element was responsive to ER stress also in this system, A549 cells transfected with pR_Δ160nt432_F, pR_ 5´Δ160nt432_F and the control plasmids, were treated with TG and the respective luciferase activities were evaluated by dual luciferase assays. The relative luciferase activity of each construct was compared to that obtained from the empty pR_F construct at the corresponding condition. Results showed that Δ160P53 IRES element is boosted from 1.47-fold to 2.32-fold over background in Vh *versus* TG-treated cells. Furthermore, a stimulatory effect of TG-treatment was also observed when the Δ160P53 5´UTR was present (Figure IV.13A). Together, these data demonstrate that synthesis of Δ160P53 is mediated by a coding region located-IRES element whose activity is inhibited by its 5´UTR and stimulated by ER stress.

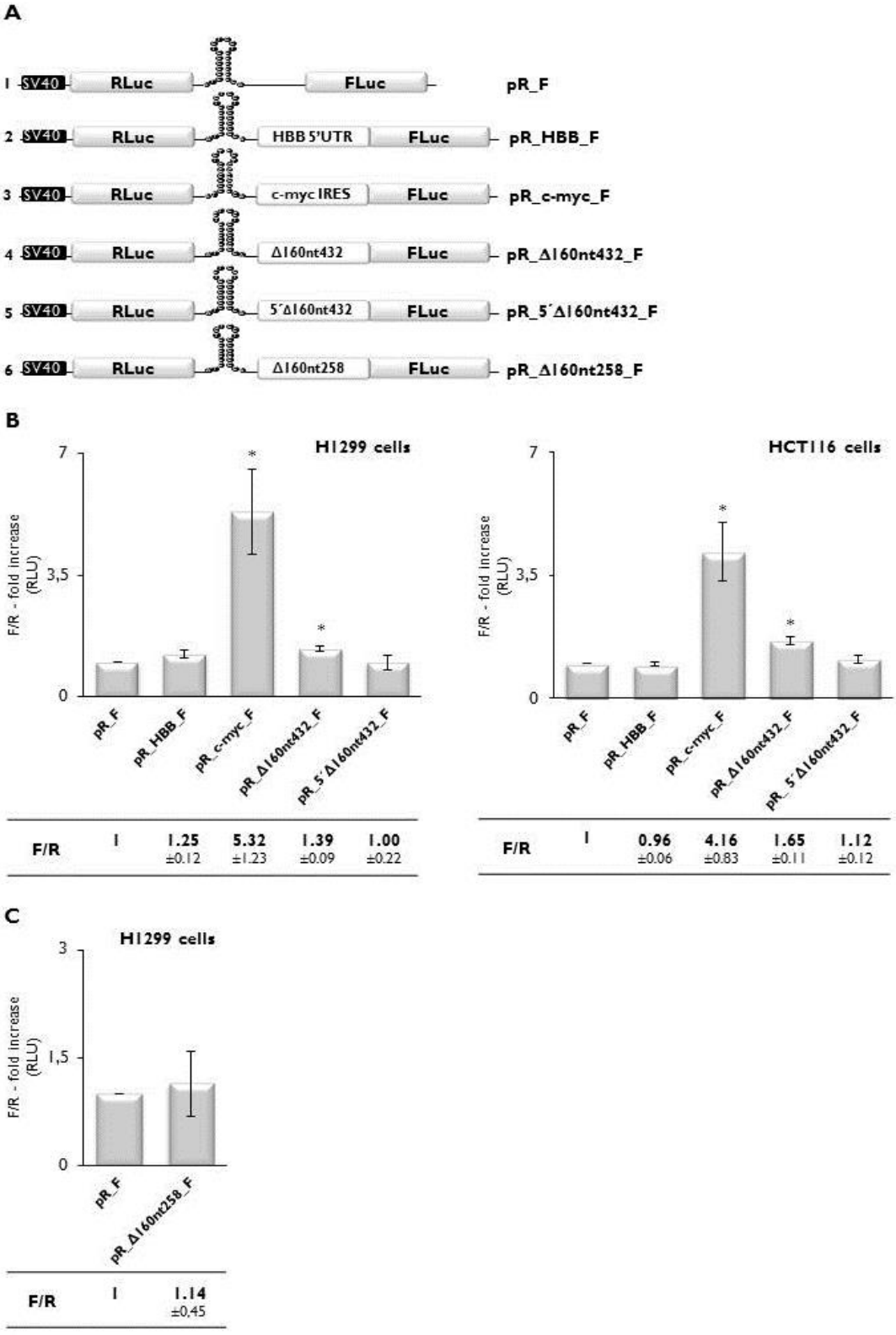
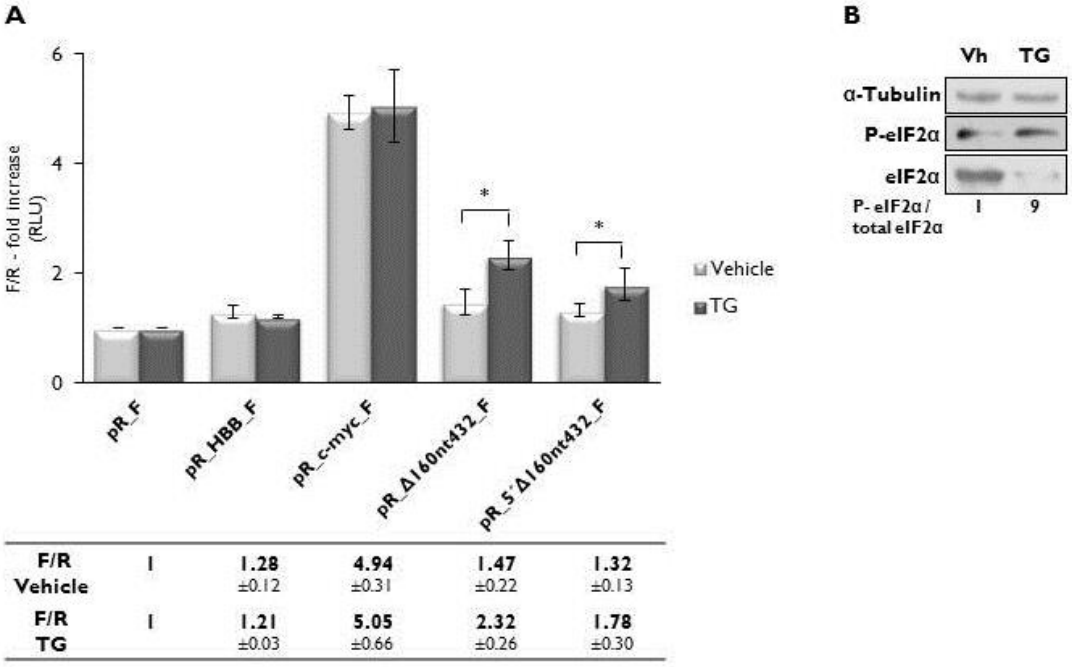


Figure IV.12. Δ160P53 coding region until nucleotide 432 induces Firefly luciferase activity in a dicistronic reporter DNA construct with impaired reinitiation and its putative 5´UTR abolishes this induction. (A) Scheme depicting the dicistronic constructs pR_F , pR_HBB_F, pR_c-myc_F, pR_Δ160nt432_F, pR_5´Δ160432_F and pR_ Δ160nt258_F. The 5' untranslated region (5'UTR) of *HBB*,

the human *MYC* IRES element and the Δ160P53 coding region until nt 432 alone or proceeded by its putative 5′UTR, as well as the Δ160P53 coding region until nucleotide 258, were cloned upstream of FLuc into the pR_F vector, downstream of the RLuc ORF (RLuc Box) and of a stable hairpin structure, but upstream of the FLuc ORF (FLuc box), to create pR_HBB_F, pR_c-myc_F, pR_Δ160nt432_F, pR_5′Δ160432_F and pR_ Δ160nt258_F constructs, respectively. The dicistronic transcriptional units are under the control of SV40 promoter. **(B)** Δ160P53 coding region until nucleotide 432 induces FLuc activity which is inhibited by its putative 5′UTR. H1299 and HCT 116 cells were transiently transfected with the dicistronic constructs pR_F, pR_HBB_F, pR_c-myc_F, pR_Δ160nt432_F and pR_5′Δ160432_F, depicted in A, and luciferase activity was measured 24 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between FLuc and RLuc, normalized to that of the empty construct, arbitrarily set to 1. Data are presented below each graph as the means ± standard deviation (SD) of at least 3 independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$. **(C)** Δ160P53 coding region until nucleotide 258 does not induce FLuc activity. H1299 cells were transiently transfected with the dicistronic plasmids pR_F and pR_ Δ160nt258_F depicted in (A) and luciferase activity was measured as in (B).



F/R Vehicle	1	1.28 ±0.12	4.94 ±0.31	1.47 ±0.22	1.32 ±0.13
F/R TG	1	1.21 ±0.03	5.05 ±0.66	2.32 ±0.26	1.78 ±0.30

Figure IV.13. Δ160P53 IRES is stimulated by endoplasmic reticulum stress in a pR/F system with impaired reinitiation. A549 cells were transiently transfected with dicistronic constructs pR_F, pR_HBB_F, pR_c-myc_F, pR_Δ160nt432_F and pR_5′Δ160432_F depicted in Figure IV.12A and treated 19 hours later with DMSO (Vh) or1μM-thapsigargin (TG) for 20 hours. **(A)** Δ160P53-driven Firefly luciferase (FLuc) activity is induced by ER stress. Luminescence assays were performed using extracts from A549 transfected cells. The values (relative light units; RLU) are shown as the luminescence FLuc/RLuc ratio, normalized to that of the

pR_F construct at each condition, arbitrarily set to 1. Data are presented as the means ± SD from three independent experiments. Statistical analysis was performed using Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$. **(B)** 1μM-TG treatment increases EIF2α phosphorylation. Western blot analysis of extracts of A549 cells transfected with pR_Δ160nt432_F construct and treated with DMSO (Vh) or 1μM-thapsigargin (TG), using antibodies against total and phosphorylated EIF2α (P-EIF2α) proteins and against human α-tubulin, for protein loading control.

IV.3.3. Increased EIF2α phosphorylation accounts for the induction of IRES-driven production of Δ160P53 upon ER stress

Increase of EIF2α phosphorylation is one of the cellular responses to ER stress (Harding et al., 1999; Harding et al., 2000a) and this condition has been associated with stimulation of activity of some IRESs, such as *MTOR* as previously shown. Accordingly, it was assessed whether increased phosphorylation levels of EIF2α might be accounting for the Δ160P53 IRES induction observed upon ER stress. For that, extracts from TG-treated A549 cells transfected with pR_Δ160nt432_F that assayed positive for Δ160 IRES activity (Figure IV.13A), were analyzed by Western Blot with antibodies against EIF2α total and phosphorylated (EIF2α) proteins. Results demonstrated that TG-treatment that stimulated Δ160p53 IRES activity also augmented phosphorylation of the EIF2 α-subunit (Figure IV.13B). These data indicate that induction of IRES-driven synthesis of Δ160P53 correlates with an increase of EIF2α phosphorylation, suggesting that the latter event is responsible for the stimulatory effect observed upon ER stress conditions on Δ160P53 IRES activity.

V. DISCUSSION AND CONCLUSIONS

V.1. Dicistronic DNA reporter – a tool to assay for IRES activity

Dicistronic DNA reporter systems are powerful tools to test IRES activity of a given sequence. The first cistron serves as an internal normalizer, since it allows corrections of transfection efficiencies. And, more importantly, it constitutes a "barrier" for ribosomes recruited to the mRNA in a cap-dependent manner, especially when followed by structured RNA sequences that prevent ribosome reinitiation events. The used dicistronic reporter harbors a stable hairpin downstream of RLuc, the first cistron. Here, it is demonstrated that this hairpin efficiently inhibits ribosome scanning, as previously shown (Figure IV.2B; Candeias et al., 2006). Furthermore, one additional stop codon is located downstream of RLuc of the dicistronic DNA plasmid, in order to prevent ribosome read-through. The AUG position relative to the IRES element might be important for internal translation initiation, considering that some IRESs directly recruit the translational apparatus to the initiation codon (eg., Kaminski et al., 1990), in addition to the fact that the overall RNA secondary structure of some IRESs elements displays an important role for translational machinery assembly (Jang and Jan, 2010; Serrano et al., 2009), whereas other IRESs have independent sequence modules coordinating ribosome recruitment (Jopling et al., 2004). Accordingly, the IRESs (and control) sequences were cloned immediately upstream of FLuc in a way that the IRES initiator is located at his native position (Figure IV.2B). One exception was applied for the putative IRES for Δ160p53, as it was anticipated that Δ160p53 coding region could play a role for the activity of this cis-regulatory element (as latter confirmed).

Expression of the second cistron from a dicistronic DNA plasmid might arise also from IRES-independent events, such as splicing activation or cryptic promoter activity of the

sequence under study. The presence of a splice acceptor site within the intercistronic region might elicit production of aberrantly spliced transcripts carrying FLuc ORF that is translated in a cap-dependent manner. Appendix 1 shows an example of how cryptic splicing events at the dicistronic R/F reporter may generate false-positive results. Insertion of a putative adenomatous polyposis coli (*APC*) IRES sequence (Goss et al., 2002) in the intercistronic region of pR/F plasmid highly increased FLuc activity (193-fold over background) (Figure VII.1), that was reduced by a stable hairpin upstream of RLuc (Figure VII.3), indicating that FLuc expression was somewhat cap-dependent. RNA integrity control by RT-PCR and sequencing, revealed a splice acceptor site within *APC* sequence eliciting production of a single aberrantly spliced mRNA (Figure VII.4) or both unspliced and spliced transcripts (Figure VII.5), according to the location of the splice donor site within RLuc ORF. One of the spliced transcripts encoded an RLuc-FLuc fusion protein, in which RLuc was C-terminally deleted, explaining the observed decrease of RLuc activity promoted by the *APC* sequence (Figure VII.2), while FLuc protein was intact. Accordingly, the high FLuc levels observed were due to cap- rather than IRES-dependent translation. A silent mutation at RLuc splice donor site abolished the aforementioned splicing event but generated other aberrantly spliced transcripts, in conjunction with the unspliced mRNA (Figure VII.4). These findings highlight the importance of discarding false positive results that might arise from cryptic splicing activation. Furthermore, it demonstrates that our approach accurately identifies cryptic splicing events. It is worth emphasizing that Goss et al. used two unrelated approaches to study *APC* IRES activity. Accordingly, one cannot exclude that the putative *APC* IRES is a "true" IRES (Goss et al., 2002).

FLuc might be also expressed in an IRES-independent manner if the DNA sequence in the intercistronic region of pR/F displays cryptic promoter activity, generating a

monocistronic transcript encoding FLuc protein that co-exist with the full-length transcript. A strategy to rule out cryptic promoter events is to assess FLuc expression from promoterless reporter vectors. If FLuc expression remains unchanged upon promoter removal, the sequence placed between the cistrons has cryptic promoter rather than IRES activity, as it occurs for *MLH1* 5´UTR (Figure IV.4). On the other hand, if FLuc expression is reduced to background levels, one can discard cryptic promoter activity of the sequence under study, as it occurs for *MTOR* 5´UTR-driven FLuc production (Figure IV.4C, D). Interestingly, transfection of promoterless plasmids in HEK293T cells originates some expression of RLuc (Figure IV.4B). Our pR/F plasmid is based on the phRL-SV40 Vector (Promega), and a study showed that this plasmid might be transcribed from non-SV40 promoter plasmid regions. This study further showed that pBS, a promoterless cloning plasmid vector, and pEGFP-C1 vector also harbor spurious transcription, demonstrating that this event is not restricted to phRL-SV40 vector (Nejepinska et al., 2012). Those findings might explain why RLuc is expressed from the promoterless plasmids in the present study. Nevertheless, it remains to be clarified why this spurious transcription only occurs in HEK293T cells. One may speculate that differences in expression of transcription factors regulating this non-SV40-triggered transcription in HEK293T comparing to HeLa and A549 cells, might account for these observations. As this spurious transcription originates transcripts encoding also RLuc protein, which is our internal normalizer, it will not affect the IRES outcome of the sequences under study. Furthermore, in this study, FLuc activity driven by *HBB* 5´UTR (negative control for IRES activity) was measured in parallel, in order to check if a non-IRES related event was affecting the measurement outcome.

Cryptic promoter activity of FLuc ORF has been reported (Vopalensky et al., 2008); in addition, removal of SV40 promoter from the empty construct diminishes FLuc expression (Figure IV.4B). Accordingly, in our system, FLuc protein expressed by the empty plasmid is also being produced by an event occurring at the dicistronic mRNA itself. This event might be ribosome reinitiation or read-through. It is considered that ribosome reinitiation is an improbable event in the used system because: (i) ribosome reinitiation after a long uORF is highly inefficient (Kozak, 2001; Luukkonen et al., 1995), since post-terminating ribosomes must carry some initiation factors to effectively reinitiate (Poyry, 2004; Szamecz et al., 2008)); (ii) a stable hairpin that reduces RLuc expression by about 60% (Figure IV.2B), was introduced downstream of RLuc ORF. It has been reported that reinitiation after translation of a 55-codon uORF, which is far shorter than the 312-codon RLuc ORF of the used DNA reporter system, is completely inhibited (Luukkonen et al., 1995). Even if some EIFs-carrying ribosomes were able to remain attached to the mRNA after RLuc translation, downstream scanning will be impaired by the stem-loop hairpin. Accordingly, although an additional stop codon is located downstream of RLuc ORF, it is considered that the most probable event contributing to FLuc expression deriving from the empty vector (besides its cryptic promoter activity) is read-through. Therefore, in HEK293T cells, taking into account that some dicistronic transcripts are still produced, even when SV40 promoter is removed, FLuc expression deriving from the empty construct reflects not only spurious transcription at FLuc, but also some ribosome read-through at the remaining mRNAs. Note that these considerations are referring to residual values of FLuc expression (deriving from the empty DNA dicistronic plasmid) and that the events occurring at this reporter plasmid (which is our normalizer) are the same observed for the dicistronic plasmid containing the putative IRES. Bearing in mind that

a plasmid carrying a negative control sequence is also subjected to the same events, those limitations will produce little (if any) effect in the obtained results. To my knowledge, it is difficult to have a perfect system.

FLuc expression deriving from a transfected dicistronic RNA reporter unequivocally discards cryptic promoter activity in the sequence under study. However, some cellular IRESs require the so-called "nuclear experience" that might be important for assembly of ITAFs (Semler and Waterman, 2008) or mRNA modifications such as pseudouridylation (Ge and Yu, 2013) and methylation (Zheng et al., 2013). A negative result for a putative IRES by directly transfecting RNA into the cytoplasm might arise from a nuclear event-dependency only. Accordingly, it is considered that RNA transfection must only be performed as a complementary approach to rule out cryptic promoter activity, unless DNA-based methods turn out to be unfeasible.

V.2. MTOR

MTOR protein kinase regulates cell growth, proliferation, autophagy, cell cycle progression and autophagy, acting as a master switch between anabolic and catabolic processes (reviewed in Laplante and Sabatini, 2012). Regulation and function of MTORC1 and MTORC2 signalling are subject to extensive studies, however little is known about MTOR protein expression regulation itself.

To cope with stress conditions, cells reduce energy consumption, by inhibiting overall mRNA translation, and direct efforts towards synthesis of stress-responsive proteins. Indeed, this is achieved namely by inactivating MTORC1 signals (reviewed in Sengupta et al., 2010). The reversibility of this inactivation is demonstrated by the fact that amino acids replenishment restores p70 S6 kinase activity of amino acid-starved cells (Hara et al., 1998). Thus, it is expected that MTOR protein expression itself is not

greatly reduced by global translational inhibitory conditions, in order to let cells bypass the adverse condition and efficiently resume its translational ability by re-activating MTORC1 signaling. In the present work, it is demonstrated that the human *MTOR* transcript harbors an IRES element and that the IRES-driven translation of *MTOR* mRNA is stimulated in stress conditions with reduced mRNA translation, but maintenance of MTOR protein levels.

V.2.1. *MTOR* 5´UTR has IRES-like features

Cross-species sequence conservation might be an indication of a regulatory function. Conservation studies show that although different species have *MTOR* 5´UTRs with different lengths (Figure IV.1A), a degree of conservation is observed. The sequence from nt -76 to -69 might play a regulatory role as it displays high similarity among all the analyzed 5´UTRs. Furthermore, the cross-species conservation of the nucleotides immediately upstream of the translational start site goes beyond the Kozak consensus sequence (Kozak, 1984, 1986a, 1987a), since it encompasses nucleotides -30 to -1 (Figure IV.1A). Secondary structure prediction suggested that the human *MTOR* 5´UTR harbors a 5´end stem-loop, SL I, (Figure IV.1B) and it has been reported that a secondary structure located nearby the 5´ terminal cap inhibits cap-translation by limiting mRNA accessibility for preinitiation complex assembly (Kozak, 1989b). On the other hand, a stem loop located in this position is important for the activity of namely the bovine viral diarrhea virus IRES element (Yu et al., 2000). In addition, it has been reported that IRES-containing mRNAs harbors longer 5´UTRs than non-IRES containing transcripts (reviewed in Baird, 2006) and *MTOR* 5´UTR is longer than the average.

The presence of elements within *MTOR* 5´UTR that usually represses cap-dependent translation and are involved in IRES-dependent translation is a good indicator of regulation of *MTOR* expression through internal initiation.

V.2.1.1. Does *MTOR* transcript have a real IRES?

The existence of an IRES element within *MTOR* 5'UTR is supported by the fact that it induces FLuc activity from a pR/F system expressed in three different cell lines (Figure IV.2). FLuc activity mediated by *MTOR* 5'UTR is neither a result of aberrantly spliced mRNAs (Figure IV.3), nor of cryptic promoter activity in the *MTOR* 5'UTR as demonstrated by the effect observed upon removal of SV40 promoter and by transfection of *in vitro* transcribed reporter RNAs (Figures IV.4 and IV.5).

As stated before, the expression of the second cistron from a dicistronic DNA plasmid may occur by a non-IRES event, such as splicing activation or cryptic promoter activity. Only a full-length unspliced dicistronic mRNA was originated by the pR_mTOR_F plasmid (Figure IV.3) demonstrating that splicing activation is not responsible for the observed FLuc induction. Removal of SV40 promoter from the pR_mTOR_F plasmid reduced *MTOR* 5´UTR-driven FLuc activity to background levels in HeLa and A549 cells (Figure IV.4 C,D). In HEK293T, the limitations of spurious transcription activation at non-SV40 promoter regions, makes evaluation of cryptic promoter activity of *MTOR* 5´UTR more challenging. *MTOR* 5´UTR-driven FLuc production is marginally reduced when SV40 promoter is removed (Figure IV.4B). Although, upon SV40 removal, RLuc is also reduced in a less extent in HEK293T cells when compared to the observed in other cell lines (Figure IV.4). It is reasoned that if FLuc induction occurred by cryptic promoter activity of *MTOR* 5´UTR, removal of SV40 promoter would had no effect on FLuc, as it occurs with *MLH1* 5´ UTR-driven FLuc (which contains a promoter).

Indeed, FLuc activity is reduced by SV40 removal (Figure IV.4B), to a similar extent than the observed in the empty plasmid (Figure IV.4A), discarding a false positive result arising from cryptic promoter activity at *MTOR* 5´UTR. Furthermore, translation of a monocistronic transcript deriving from spurious transcription at *MTOR* 5´UTR would be reduced by cap-mediated translational inhibitory conditions. In opposition to that, *MTOR*-driven FLuc activity is stimulated by ER stress, hypoxia and rapamycin treatment, with associated reduction of RLuc translation (Figures IV.6- IV.7). The fact that *MTOR* 5´UTR-driven FLuc induction is also observed upon RNA transfection (Figure IV.5) further confirms that cryptic promoter activity of *MTOR* 5´UTR is not responsible for FLuc induction. On the other hand, a higher rate of ribosome read-through or increased efficiency of translation reinitiation at FLuc AUG in the *MTOR*-containing transcript comparing to the empty counterpart could also explain the observed results. It has been demonstrated that the nucleotides surrounding the stop codon play a role in termination efficiency (Brown et al., 1990; Cassan and Rousset, 2001; Tate et al., 1996). Moreover, the nucleotide immediately upstream of the stop codon determines read-through efficiency (Cassan and Rousset, 2001. The nucleotide context of the *MTOR* 5´UTR-containing plasmid is equal to the empty plasmid. Both have an additional stop codon and a stable hairpin downstream of the RLuc ORF. Hence, differences in read-through events between the empty and *MTOR* 5´UTR-containing transcripts are highly improbable.

As previously stated, ribosome reinitiation at FLuc AUG is improbable due to RLuc ORF length and constraints of the stable hairpin located in the intercistronic space. Nevertheless, assuming that a few subsets of ribosomes are able to retain some EIFs during RLuc translation and overcome the scanning inhibitory effect caused by the stem-loop hairpin, a decrease on the availability of functional TCs due to increased

phosphorylation of EIF2α would reduce translation reinitiation efficiency or, since the intercistronic region is relatively long, would have no effect. On the contrary, a stimulation of *MTOR* 5´UTR-driven FLuc activity is observed in conditions with associated EIF2α phosphorylation (Figures IV.6 and IV.7). The existence of a uORF in the intercistronic region that fails to be translated under stress conditions, leading to enhanced reinitiation of FLuc when TCs are low, could explain these observations. This would imply that, under stress conditions with low functional TCs, the ribosome, after translating a long uORF, would bypass a given uORF in the intercistronic region and reinitiate at FLuc AUG, liberating the uORF-mediated repression of FLuc translation reinitiation. However, this would not explain why FLuc expression is induced by *MTOR* 5´UTR under unstressed conditions. As observed for *ATF4* (Vattem and Wek, 2004) and other examples not mentioned in this thesis, uORFs with different lengths, located at different positions relative to downstream ORF AUG or upstream ORF stop codon might determine main ORF expression in several different ways, both under unstressed and stress conditions. Nevertheless, the inhibitory effect of a hairpin on translation reinitiation is dual: it inhibits ribosome scanning (and reinitiation is dependent on scanning) and, if a uORF is being translated, it pauses translation elongation which leads to dissociation of EIFs (Kozak, 1986b, 2001). On the other hand, the pausing of a scanning ribosome could grant more time to the re-acquisition of TCs. However, this effect would also result in translation reinitiation at FLuc AUG at the empty and *HBB*–containing transcripts, which is not observed. Accordingly, translation reinitiation at FLuc AUG in the *MTOR* 5´UTR-containing transcript is unlikely to occur. Hence, it is considered that the observed *MTOR* 5´UTR-driven FLuc activity is neither a result of an RLuc translation-dependent event, nor splicing

activation or even due to production of aberrant monocistronic transcripts due to cryptic promoter activity at *MTOR* 5´UTR.

Here it is shown that *MTOR* IRES activity varies between different cell lines and the same effect is observed for the cellular and viral IRESs controls. The present data show that the activity of *MYC* IRES is greater in HeLa than in HEK293T cells, which is in accordance with previous reports (Stoneley et al, 2000b). However, a greater activation of EMCV IRES in HeLa than in HEK293T cells is observed, while Creancier et al. (2001) reported the opposite (Creancier et al., 2001). These discrepancies might be due to different features of the pR/F system used in both studies: in this study, a stable hairpin was inserted downstream of RLuc cistron into the empty, negative control and IRES-containing reporters in order to have similar levels of translation reinitiation inhibition in all constructs; on the contrary, Creancier et al. (2001) introduced an hairpin only in the empty construct (Creancier et al., 2001). This and other differences in the characteristics of the used dicistronic reporter systems might be responsible for the different results.

Comparing the activity of *MTOR* IRES with that from the positive controls IRESs used, it is observed that in HEK293T cells, its activity is similar to that observed from *MYC* IRES and greater than that from EMCV IRES element (Figure IV.2); in addition, it has been demonstrated that *MYC* IRES is just 3-fold less active than its cap-dependent translation (Stoneley et al., 2000b). These results suggest that *MTOR* IRES activity is robust and might account for the maintenance of MTOR protein levels under stress conditions with associated overall protein synthesis inhibition.

Like cap-dependent translation, IRES-mediated translation requires a battery of proteins for effective recruitment of the translational machinery that might include canonical initiation factors and/or ITAFs. The presented data indicate that *MTOR* IRES

activity is neither dependent on functional ternary complexes, as it is resistant to an increase of EIF2α phosphorylation, nor on EIF4F complexes, since it is still functional when correct formation of EIF4F complexes is disrupted by inactivation of MTORC1 signaling. The phosphorylation state of 4EBP1 was not addressed in this study and it has been demonstrated that 4EBP1 might regain its phosphorylation upon prolonged treatment with rapamycin (Choo et al., 2008). Nevertheless, in this study, cells were exposed to this macrolide only for 6 hours (Figure IV.8). The fact that, 4EBP1 phosphorylation might be only moderately decreased upon rapamycin treatment (Thoreen et al., 2009) in combination with the fact that this MTORC1-phosphorylation target is the master effector of MTORC1 signalling in the regulation of protein synthesis (Hsieh et al., 2012; Thoreen et al., 2012), might explain why treatment with rapamycin only had a little effect on protein synthesis (Figure IV.8). Nevertheless, if IRES-driven translation of *MTOR* required functional EIF4F complexes, even a slight change in the 4EBP1 phosphorylation levels would have a negative effect on its activity, which is not observed. In addition, S6K1 is known to phosphorylate PDCD4, inducing its degradation (Dorrello et al., 2006). PDCD4 inhibits EIF4A-EIF4G interaction (Yang et al., 2003), further suggesting that *MTOR* IRES activity is independent of EIF4F complexes and, consequently, of ribosomal scanning. The RNA helicase activity of EIF4A is particularly important for scanning of highly structured 5'UTRs (Svitkin et al., 2001). Furthermore, the role of S6K1 on EIF4A also relies on its ability to phosphorylate EIF4B (Raught et al., 2004; Shahbazian et al., 2006), that stimulates the RNA helicase activity of EIF4A (Abramson et al., 1988; Lindqvist et al., 2008; Rogers et al., 1999, 2001). Actually, conditions with low ternary complex and EIF4F levels stimulated *MTOR* IRES activity (Figures IV.6- IV.8), suggesting that the competitive

fitness of *MTOR* transcript for binding the translational machinery is potentiated in comparison to the cap-dependent translated mRNAs.

It has been suggested that the intracellular concentration of ITAFs plays a role in modulating IRES activity (reviewed in Lewis and Holcik, 2007), which might also account for the stimulatory effect triggered by the stress conditions used in this study. Furthermore, it can explain the different levels of activation of *MTOR* IRES obtained across the three cell lines. Many ITAFs shuttle between the nucleus and the cytoplasm (Michael et al., 1997; reviewed in Piñol-Roma, 1997); in addition, it has been suggested that the experience of different subcellular compartments (nuclear and cytoplasmatic) of IRES-containing mRNAs determines the assembly of different ITAFs to the mRNP (reviewed in Lewis and Holcik, 2007). Based on these data, transfection of reporter RNAs was performed, showing that *MTOR* IRES activity is not dependent on a nuclear event, but might be stimulated by it, since the levels of *MTOR* IRES activation obtained in the RNA transfection assays are lower than those observed when cells were transfected with the reporter DNA constructs. One might speculate that the *MTOR* mRNP is composed of predominantly cytoplasmatic ITAFs but the assembly of a nuclear protein or a protein that shuttles to the nucleus might be beneficial. Furthermore, the cytoplasmatic redistribution of *MTOR* ITAFs upon $CoCl_2$-induced hypoxia, TG-triggered ER stress or rapamycin-driven MTORC1 inactivation might also account for the observed *MTOR* IRES stimulation. A similar event occurs for *BAG1* IRES-driven translation that is sustained in chemotoxic stress due to cytoplasmatic relocalization of the *BAG1* ITAFs PTB and PCBP1 (Dobbyn et al., 2008).

V.2.2. *MTOR* IRES is stimulated by stress conditions

Hypoxic and ER stress conditions orchestrate a series of signals that decrease protein translation and initiate a translational reprogramming (Arsham, 2003; Harding et al., 1999; Harding et al., 2000a, 2000b; Koritzinsky et al., 2006, 2007; Koumenis et al., 2002; Liu et al., 2006). One mechanism by which hypoxia reduces protein synthesis is through activation of the UPR branch triggered by PERK stimulation (Koritzinsky et al., 2006, 2007; Koumenis et al., 2002; Liu et al., 2006). This branch leads to phosphorylation of EIF2α, which allows selective translation of transcripts encoding proteins involved in stress response (reviewed in Wek et al., 2006), namely through IRES or uORF elements. One example of selective protein synthesis is the EIF2α phosphorylation-dependent translation of the transcript encoding ATF4, that is important for the cellular response to ER stress (Blais et al., 2004). Under normal conditions, efficient translation of *ATF4* is impaired by two uORFs within its 5'UTR, whereas conditions with reduced levels of ternary complexes favor reinitiation at the main start codon by decreased translational efficiency of the second uORF (Vattem and Wek, 2004). Furthermore, an alternatively spliced variant of *ATF4* is translated via an IRES element which is activated by PERK-induced EIF2α phosphorylation (Chan et al., 2013). Additionally, the mRNA translation of *HIF1α*, a major coordinator of the hypoxic response, is maintained in hypoxia through an IRES element (Lang et al., 2002). The findings presented here demonstrate that *MTOR* IRES activity is potentiated by hypoxia and UPR with increased levels of phosphorylated EIF2α that might account for maintenance of MTOR protein expression. Although other mechanisms might stimulate *MTOR* IRES, our results suggest that EIF2α phosphorylation may be, at least partially, involved in that activation, as (i) hypoxic conditions only increase relative *MTOR* IRES activity when EIF2α is phosphorylated, independent of HIF1α stabilization,

and (ii) *MTOR* IRES activation during TG-induced UPR is more evident when EIF2α is phosphorylated. It has been shown that MTOR is involved in the hypoxic response namely as it potentiates *HIF1α* mRNA translation and modulates HIF1α-dependent transcriptional induction (Bernardi et al., 2006; Hudson et al., 2002; Land and Tee, 2007; Laughner et al., 2001; Thomas et al., 2006; Zhong et al., 2000). Furthermore, MTORC1 signaling is inactivated by hypoxia through HIF1α-independent and dependent mechanisms, particularly when in conjunction with other stresses (reviewed in Wouters and Koritzinsky, 2008). It has been suggested that severe exposure to hypoxia is characterized by a biphasic inhibition of mRNA translation in which the first phase (acute response) of inhibition is achieved by PERK-EIF2α activation, which switches to MTORC1- and 4ET- mediated protein synthesis reduction (Koritzinsky et al., 2006). The obtained results suggest a new layer of *MTOR* regulation in hypoxia. It is tempting to speculate that enhanced IRES activity of *MTOR* is a cellular attempt to maintain MTORC1 functional in the acute response to hypoxia by creating a positive feedback loop in which hypoxia-induced EIF2α phosphorylation activates *MTOR* IRES, which in turn, aids HIF1α stabilization. Actually, it has been suggested that the degree of MTORC1 inactivation under hypoxia is not enough to reduce HIF1α protein expression (Thomas et al., 2006). The *MTOR* IRES might be aiding this incomplete inactivation.

MTORC1 signalling and ER stress-triggered UPR reciprocally regulate each other and, in particular, by activating the IRE1-JNK axis of UPR, MTORC1 induces apoptosis upon ER stress (Kato et al., 2011, 2013; Ozcan et al., 2008), so it is not surprising that *MTOR* is one of the transcripts that is preferentially translated during UPR. Indeed, it is shown that *MTOR* IRES is largely stimulated by UPR. Thus, it was hypothesized that a

synergistic effect between increased phosphorylation of EIF2α and pro-apoptotic signals might be responsible for this substantial stimulation of *MTOR* IRES activity. Indeed, it is observed that a stimulation of *MTOR* IRES activity also occurs in UPR with unphosphorylated EIF2α, but to a lesser extent. However, the maintenance of EIF2α phosphorylation parallels the anti-apoptotic response of ER stress. Thus, another UPR-mediated signal might also be triggering *MTOR* IRES activity. One cannot exclude that this signal is dependent on PERK activation, since EIF2α may be unphosphorylated despite activation of the PERK branch (Novoa et al., 2001). The list of genes with translational advantage in response to MTORC1-inactivating conditions is growing (Thoreen et al., 2012) and the data presented here suggest that *MTOR* is one of those genes. Stimulation of *MTOR* IRES by treatment with rapamycin is consistent with a previous study demonstrating that some IRES-containing transcripts are translationally upregulated by MTORC1-inactivating conditions (Thoreen et al., 2012). Particular stimulation of *MTOR* IRES activity by MTORC1 inactivation suggests a feedback loop to maintain MTOR protein levels.

A complex interplay between UPR- or hypoxia- and MTOR-triggered signals has been reported. The fact that the former conditions stimulate IRES-driven *MTOR* translation envisions an extra layer of interconnection between these pathways. Furthermore, MTORC1-inactivating conditions also stimulate mRNA translation of *MTOR* through its IRES element. These findings suggest that stimulation of *MTOR* IRES-mediated translation is a cellular response to stress conditions. If translation of *MTOR* would only occur via the cap-dependent mechanism, MTOR levels could be decreased by translational inhibitory conditions, leading to reduced levels of available MTORC1 and MTORC2, thus compromising cellular homeostasis. Instead, our results support a model in which IRES-mediated translation of *MTOR* mRNA contributes to MTOR

protein expression maintenance in cellular stress conditions that is able to assemble the MTORC1 and MTORC2 (independent of their activation status), either contributing to a proper stress response and/or allowing cells to restore normal growth and proliferation after bypassing the translational adverse condition (Figure V.1). Nevertheless, it could also contribute to aberrant constitutive activation of MTOR pathway and potentiate development of MTOR-associated diseases. The study presented here proposes *MTOR* IRES as a new therapeutic target for treatment of diseases with aberrant hyper-activation of MTOR mediated pathways, such as cancer.

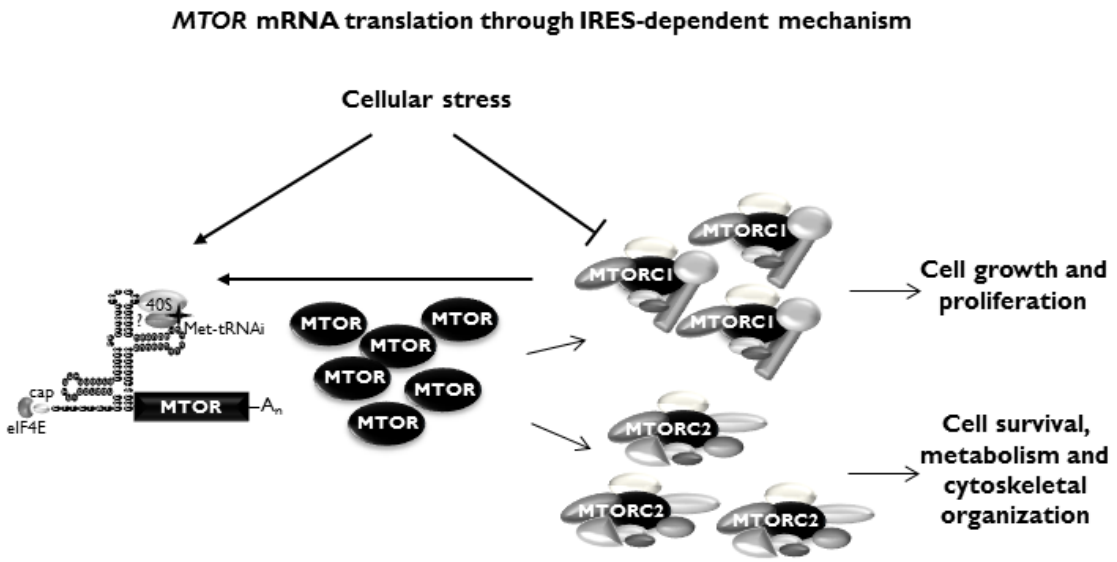


Figure V.1. *MTOR* IRES-driven translation contributes to MTOR protein expression maintenance in cellular stress conditions. Our results support a model in which IRES-driven *MTOR* translation regulation integrates the program of translational reconfiguration towards synthesis of stress-responsive proteins, triggered by adverse conditions. IRES-mediated translation of *MTOR* mRNA contributes to maintenance of expression of MTOR protein that is able to assemble the MTORC1 and MTORC2. By this mechanism, cells guarantee that MTORC1 and MTORC2-mediated signals are not lost upon translational inhibitory conditions.

V.3. Δ160P53

P53 protein has a pivotal role in suppressing tumorigenesis, namely by induction of cell-cycle arrest and apoptosis of stressed cells (reviewed in Zilfou and Lowe, 2009). When the stress condition induces a manageable cellular damage, P53 activates a program that lead to cell cycle arrest at the G1 and G2 phases (eg., Stewart et al., 1995). Nevertheless, when the damage is severe and cells acquire transforming abilities, P53 triggers senescence or induces cell death through apoptosis or autophagy (reviewed in Vousden and Prives, 2009). As a consequence, a mutation in *TP53* gene generally triggers tumor development and, actually, *TP53* is the most common mutated gene in human cancer (reviewed in Rivlin et al., 2011). Nevertheless, the behavior of tumors with deregulation of P53 activity is not predictable (eg., Ahrendt et al., 2003) and several opposing roles have been ascribed to the P53 signalling (reviewed in Vousden and Prives, 2009). The discovery of P53 isoforms, that display both P53-dependent and independent functions (Bourdon et al., 2005), has partially resolved this mystery. For instance, P53β protein isoform oligomerize with P53 and modulates P53-mediated replicative cellular senescence and apoptosis (Fujita et al., 2009). Furthermore, it has been shown that this isoform induces apoptosis in a P53-independent manner (Bourdon et al., 2005). On the other hand, Δ133P53 inhibits apoptosis triggered by P53 through reconfiguration of P53-mediated gene expression (Aoubala et al., 2010; Bourdon et al., 2005; Chen et al., 2009). These findings highlight that P53 isoforms are important modulators of P53 activity. Recently, a new P53 isoform (Δ160P53) arising from mRNA translation initiation at codon 160 from the *Δ133P53* transcript has been discovered (Marcel et al., 2010b). Notwithstanding, the regulatory mechanism governing its expression has not been studied. Here, it is demonstrated that Δ160P53 expression is translationally regulated by an IRES element

whose activity is stimulated upon stress conditions. It is further shown that this cis-regulatory element is located downstream of AUG160, and that its activity is inhibited by Δ160P53 5´UTR but induced by EIF2α phosphorylation.

V.3.1. Δ160P53 expression regulation upon stress conditions

Specific mRNA translation regulation of P53 products upon stress conditions has been reported. For instance, genotoxic stress forces expression of the Δ40P53 isoform, through increased mRNA translation of a mutant *TP53* transcript, which contributes to genotoxic-induced urinary bladder tumors (Melis et al., 2011). Here it is shown that ER stress induces Δ160P53 protein expression both in P53-expressing and -null cell lines. Furthermore, overconfluency, a stress that inhibits overall protein translation (Gerlitz et al., 2002), also increased Δ160P53 protein levels. In ER stressed A549 and HCT116 cells endogenous Δ160P53 protein induction was accompanied by reduction or maintenance of Δ133P53 protein isoform, respectively. Overconfluent A549 cells expressed similar endogenous levels of the Δ133P53 and Δ160P53, whilst in O-C NIH3T3 expressed Δ160P53 but not Δ133P53 protein isoforms (Figure IV.10A, B).

V.3.2. Δ160P53 is originated through IRES-driven translation

It has been demonstrated that IRES-mediated translation plays a role in the regulation of P53 protein isoforms. An IRES element within *TP53* transcript 5´UTR governs its translation and the Δ40P53 isoform is a result of IRES-mediated translation initiation at codon 40 (Candeias et al., 2006; Ray et al., 2006; Yang et al., 2006). The data presented here adds another IRES element governing P53 protein isoforms. The Δ160P53 IRES element is active in two different dicistronic DNA reporter vectors (Figures IV.11- 13) and its stimulation accompanies Δ160P53 endogenous protein induction. The activity

of the IRES for Δ160P53 in the pR/F reporter is relatively low when compared to that of *MYC* (Figure 2C-F) and of *MTOR* (Figure 19B, 20A) IRESs, which could indicate that the mRNA translation efficiency of this IRES element might be low. Although, one must note that the FLuc expressed from the Δ160P53 IRES-containing plasmid is fused with Δ160P53 N-terminal. Thus, the low relative FLuc/RLuc ratio observed might reflect a reduced enzymatic activity of this FLuc-Δ160P53 fusion protein, instead of inefficiency of the IRES for Δ160P53. Actually, a robust Δ160P53 protein expression deriving from the pE_Δ160_ORF dicistronic reporter is observed (2A-B). In addition, although the endogenous expression of Δ160P53 protein is low, it is similar or even greater than that observed for the cap-mediated translated Δ133P53 (Figure IV.10). Actually, in overconfluent NIH3T3 cells, Δ160P53 protein is expressed at higher levels than P53 (Figure IV.10B; right panel).

Mechanistically, the IRES element for Δ160P53 has three particular features: it is inhibited by its putative 5´UTR, it is stimulated by EIF2α phosphorylation and it is located within Δ160P53 coding region. The two latter features are not exclusive from this IRES element, since *MTOR* IRES described in this thesis is also stimulated by EIF2α phosphorylation and the IRES element that allows cap-independent production of the 5-kDa replication-associated protein 1 (RAP1) is located within the mRNA coding region (Jaag et al., 2003). Furthermore, interestingly, the IRES commanding Δ40P53 synthesis also promotes translation of P53 (Candeias et al., 2006). The fact that the RNA sequence harboring IRES activity is downstream of Δ160P53 AUG might mean that this IRES element relies on the "land and start" rather than on the "land and scan" mechanism, since backward scanning is an inefficient mechanism and only occurs over a course of very few nucleotides (Matsuda and Dreher, 2006; Kozak, 1991). The structure formed by the Δ160P53 IRES might allow a direct binding of the translational

machinery to the AUG, as it occurs with EMCV IRES element (Kaminski et al., 1994). Indeed, in line with this work, it was found that a probable secondary structure of Δ160P53 IRES positions the AUG in an accessible loop (Candeias MM, unpublished data).

Furthermore, the induction of the activity of the IRES for Δ160P53 upon conditions with low TC levels might mean that another initiation factor is responsible for the Met-tRNAi delivery to the 40S ribosomal subunit, as occurs with HCV and XIAP IRESs (Dmitriev et al., 2010; Kim et al., 2011; Skabkin et al., 2010; Thakor and Holcik, 201) or even that this IRES element operates in a Met-tRNAi-independent manner, as for IGR IRESs (Costantino et al., 2008; Wilson et al., 2000a).

To our knowledge no other IRES element is inhibited by an upstream sequence. It is possible that this inhibition relies on structural destabilization of Δ160P53 IRES by its 5´UTR. It is interesting to note that two hotspots of synonymous cancer mutations (Strauss, 2000) are located within Δ160P53 IRES element and its 5´UTR. Unpublished data from Candeias et al. (Kyoto University, Japan) actually show that a synonymous mutation at codon 151, thus within Δ160P53 5´UTR, changes Δ160P53 IRES secondary structure and strongly suggests that it abolishes the inhibitory IRES-5´UTR interaction (Candeias et al., unpublished data).

V.3.3. Activity of the IRES element for Δ160P53 upon ER stress

In the onset of ER stress, P53 is sequestered and inactivated in the cytoplasm (Pluquet et al., 2005; Qu et al., 2004) whereas prolonged or severe ER stress induces P53 expression, nuclear redistribution and transcriptional activity, which is important for ER stress-induced apoptosis (Lin et al., 2012). Furthermore, the involvement of P53 products in the ER stress-triggered apoptosis has been demonstrated by the fact that

Δ40P53 induces the expression of the pro-apoptotic proteins PUMA and PMAIP1 and is capable of apoptosis induction on ER stress in a P53-independent manner (Bourougaa et al., 2010). This isoform is induced by PERK-dependent stimulation of its IRES element, which leads to induction of 14-3-3σ, G2 arrest and suppression of P53-mediated G1 arrest (Bourougaa et al., 2010). The Δ40P53 functions are mainly dictated by the detention and absence of P53 protein domains. It retains TADII and the C-terminal OD, but lacks the N-terminal MDM2-binding site. Accordingly, it is able to regulate gene expression and form complexes with P53 with altered stabilities and functions (Candeias et al., 2006; Courtois et al., 2002; Ghosh et al., 2004; Powell et al., 2008; Yin et al., 2002).

Here it is shown that ER stress induces Δ160P53 isoform. The fact that this isoform retains the OD domain might mean that, at least in part, this isoform might be involved in the ER stress response in a P53-dependent manner. Although, curiously, unpublished results from Candeias et al. (Kyoto University) demonstrate that, at least part of Δ160P53 isoform functions, are P53-independent (Candeias et al., unpublished data). In addition, it is anticipated that Δ160P53 stability is regulated differently from P53 and similarly to Δ40P53, as it also lacks the MDM2-binding site.

The results presented here demonstrate that Δ160P53, a short isoform of P53, is stimulated upon stress conditions by induction of a coding region-located IRES element. It is demonstrated that EIF2α phosphorylation, which mediates overall cap-dependent translation inhibition in several stress and physiological conditions, is the most probable responsible event for this induction. Actually, it seems that mRNA translation through IRES elements contributes to the orchestration of the P53 network of proteins, upon different conditions. The fact that the IRES element for Δ160P53 and its inhibitory 5´UTR are in a region that is frequently mutated in cancer,

might suggest an important role for this isoform in tumour development. Actually, results from our collaborator group demonstrate that the Δ160P53 protein isoform is an oncogene (Candeias et al., unpublished data).

V.4. IRES and cancer

Here, an IRES element within *MTOR* transcript was identified, and the involvement of the MTOR signalling in tumorigenesis is well known. In addition, the presented results demonstrate that the oncogenic Δ160P53 protein isoform is also synthesized through IRES-mediated translation. To expand our knowledge about the involvement of IRES-driven translation in tumorigenesis, it was assessed whether colorectal cell transformation and metastization might be accompanied with global alterations in translation mediated by such cis-regulatory elements. It was observed that both cellular IRESs (*MYC* and *MTOR*) behave similarly, in a way that their activity is stimulated by cellular transformation to a malignant phenotype, which is reversed by malignization. On the other hand, the activity of EMCV IRES is only stimulated in the metastatic cell line (Figure IV.9). It has been demonstrated that overexpression of EIF4E contributes to the initial burst of protein synthesis observed in colon carcinogenesis (Rosenwald et al., 1999) and an increase in its activity is essential for maintaining transformation and induction of motility and metastization of colorectal cancer cells (Ye et al., 2013). As brought up above, it has been proposed that EIF4E might actually act as a cap- to IRES-mediated translation switcher (Svitkin et al., 2005). Furthermore, it has been suggested that the expression of other initiation factors is deregulated in CRC, such as of EIF2α, that seems to play a role in tumor initiation and progression (Rosenwald et al., 2003). The data presented here suggest that deregulation of initiation factors activity during CRC tumorigenesis does not account

per se for the activation status of general IRES-mediated translation. Those observations corroborate the fact that the mechanism of action of different IRESs varies significantly, and that the activation level of different IRES entities is differentially affected by canonical initiation factors. The stimulation of IRES elements is also dependent on ITAFs and actually, it has been demonstrated that differential alterations in the expression and subcellular localization of hnRNPs are observed in primary and metastatic colorectal cancer (Hope and Murray, 2010).

One cannot exclude that in the used settings, a global mechanism might be affecting the entire set of cellular IRESs differently from that of viral IRESs, as both *MYC* and *MTOR* IRESs behaved similarly during CRC malignant transformation and metastization, in opposition to what happens to EMCV IRES. Nevertheless, it is more likely that the different requirement of initiation factors for ribosome recruitment (both canonical and non-canonical), dictate specific stimulation of a certain IRES element under different physiological or pathophysiological conditions.

V.5. A note about IRES-mediated translation

The translational apparatus of IRES-mediated translation relies on a variety of canonical initiation factors that might participate through canonical and non-canonical interactions; as well as accessory proteins that are involved in other biological processes which are not related to the "standard" mechanism of translation initiation involving cap recognition. The mRNP composition of viral IRESs allows their classification in different groups and types of IRESs, while it is highly variable for cellular IRESs (reviewed in Martínez-Salas et al., 2012). No common structural motif is found in cellular IRESs in opposition to viral IRESs that are highly conserved structures with defined functions (Honda et al., 1996; Martınez-Salas, 2008). The only primary

sequences identified until now to have a role in cellular IRES activity are the polypyrimidine sequences, for PTB binding (Mitchell et al., 2005) and poly(A) tracts for PAB1 binding (Gilbert et al., 2007).

The independence of EIF4E seems to be the most universal feature of the battery of IRESs identified until now (with the exception of some aforementioned viral IRESs) (reviewed in Hellen and Sarnow, 2001). Furthermore, the independence of EIF2 seems to be a feature of several IRESs, as demonstrated by the growing list of transcripts whose IRES-driven translation is not impaired by conditions with associated increase in phosphorylation of EIF2α (Allam and Ali, 2009; Fernandez, et al., 2002; Gerlitz et al., 2002; Thakor and Holcik, 2011). It has been demonstrated that a possible explanation for this independence might rely on the substitution of EIF2 function by utilization of other initiation factors, combination of initiation factors or IRES structural mimicking of Met-tRNAi to directly assemble the 80S ribosomes (Costantino et al., 2008; Dmitriev et al., 2010; Kim et al., 2011; Pestova et al., 2008; Skabkin et al., 2010; Thakor and Holcik, 2011).

The mechanism of action of IRES-mediated translation might prompt one to wonder if it represents an evolutionary adaptation to adverse conditions. On other hand, considering that it implies a simpler mean to initiate translation, IRES-driven translation might be a reminiscent of the mechanism of translation initiation used by the eukaryotic ancestors, that remained conserved upon evolution in order to allow an effective response to demanding conditions. It seems quite logical to suppose that IRES-driven translation is an ancient form of translation that evolved into the more complex cap-dependent mechanism, though remaining the main mechanism of translation during stress. Accordingly, oscillations between normal and stress conditions either favored the canonical or the IRES-dependent mechanism,

respectively. The more ordered and universal rearrangement of proteins and interactions observed in the canonical mechanism was achieved through extended periods of normal conditions (i. e., unstressed conditions), leading to the generation of an equilibrated mechanism. On the other hand, the more disperse and variable battery of initiation factors participating in IRES-dependent translation was achieved through selective pressure under stressed conditions, using proteins rather than EIFs; or EIFs with unusual interactions or functions. These assumptions might be substantiated by the discovery of IRESs elements that function in yeast, insects, plants and mammals (Dorokhov et al., 2002; Woolaway et al., 2001). Furthermore, the cap structure and the cap-binding protein EIF4E are only used by eukaryotes (Marcotrigiano et al., 1997; Matsuo et al., 1997). In addition, the canonical mechanism allows translation with higher efficiency in comparison to IRES-dependent translation in normal conditions, although the latter mechanism allows efficient mRNA translation upon adverse conditions. These observations suggest that cap-dependent translation is an evolutionary update of the pre-existed IRES-mediated translation. Some similarities with prokaryotic translation or translational control mechanisms are observed for some IRESs elements, such as those found in the transcripts of the human NK6 homeobox 2 and insulin-like growth factor 1 receptor. They are very small, nine and nineteen nts, respectively, and base pair with 18S rRNA, similarly to the prokaryotic Shine-Dalgarno interaction with 30S ribosomes (Chappell et al., 2004; Meng et al., 2010). Furthermore, *CAT1* IRES activity is stimulated by ribosome stalling during translation of an uORF, which is similar to the mechanisms controlling prokaryotic transcription and translation (Fernandez et al., 2005; Lee and Yanofsky, 1977; Narayanan and Dubnau, 1987).

The vast majority of cellular IRESs assist translation with a poorer efficiency comparing to the viral counterparts (reviewed in Komar and Hatzoglou, 2005). The fact that the vast majority of viral mRNAs does not possess the cap structure and are translated through highly organized IRES elements (eg., Honda et al., 1996), might indicate that the IRES-mediated translation of viral transcripts is an evolved version of this mechanism of translation, and resulted from the evolution of a single mechanism of translation.

If this theory proves to be accurate, within years, due to the constant stress conditions that we force our cells to be exposed to, a dissertation about IRES-dependent translation will substitute affirmations as "inefficient IRES-mediated translation" by "IRES-dependent translation as efficient as cap-dependent translation" or at least "the cellular and viral IRES-mediated translation efficiency is similar".

VI. CONCLUDING REMARKS AND FUTURE DIRECTIONS

My original contribution to knowledge is that human *MTOR* transcript has an IRES element that (i) does not require nuclear experience to be active; (ii) is induced by hypoxia only when the phosphorylation levels of EIF2α are augmented, and not when HIF1α protein is stabilized *per se*; (iii) is stimulated by UPR mainly in the pro-apoptotic phase with low TC levels and (iv) is potentiated by MTORC1 signalling inhibition. In addition, it is demonstrated that Δ160P53 protein isoform is induced, by augmented translational rates, in UPR and stress driven by cellular over-confluency and that an IRES element within *Δ133P53* transcript is responsible for this induction. This IRES element is inhibited by Δ160P53 5´UTR and stimulated by EIF2α phosphorylation.

The vast majority of IRES elements are located within the 5´UTR of transcripts, although some cases of hybrid 5´UTR-coding region IRESs have been reported (Allam and Ali, 2009; Candeias et al., 2006) By extending the analysis of *MTOR* 5´UTR in terms of secondary structure prediction, through submission of longer sequences of *MTOR* mRNA to M-Fold program (Zuker, 2003), it was realized that it is possible that *MTOR* coding region might play a role in IRES-mediated translation. A representative structure of this analysis is shown in Figure VI.1. This structure folds with a Gibb´s Free Energy of -63.33 kcal/mol and besides the previously predicted SL I, it harbors another individual stem-loop, SL II, and a composed secondary structure consisting of stem-loops III and IV (ΔG = -37.03 kcal/mol) (Figure VI.1). As previously mentioned, the sequence from nt -76 to -69 has cross-species conservation (Figure IV.1A) and in the present prediction, this sequence forms the stem from SL II (Figure VI.1). In addition, the other highly conserved sequence of *MTOR* 5´UTR corresponds to SL III and part of SL IV (Figure IV.1A and Figure VI.1). Those findings might indicate that SL II, SL III and SL IV form naturally. Furthermore, in this prediction, the composed

secondary structure has a Y-like shape and resembles the Y-type structural motif found in *TMEM132A*, *FGF2* and picornavirus IRESs (Le and Maizel, 1997). Moreover, the *MTOR* initiation codon is located in the apical loop of SL IV (Figure VI.1) and thus, is accessible for the translational machinery. Accordingly, it is tempting to speculate that SL III and SL IV contribute to AUG presentation to the translational machinery. The 5´ terminal of human *MTOR* 5´UTR is not present in *MTOR* transcripts from all the analyzed species (Figure IV.1A), suggesting that it does not play an essential role for *MTOR* IRES activity. Therefore, SL I can either function as a non-essential enhancer of *MTOR* IRES activity, such as the 75-nts hairpin of HCV IRES (Reynolds et al., 1995) or it can rather serve to inhibit the canonical cap-dependent mechanism. It would be of full interest to verify the aforementioned predictions, by determination of *MTOR* IRES structure through, namely, chemical and enzymatic probing. It has been observed that IRES-mediated translation might depend on the overall RNA structure, in which different stem loops and pseudoknots cooperatively function to recruit the translational apparatus (Jang and Jan, 2010; Serrano et al., 2009). This is particularly true for viral IRESs. Cellular IRESs most commonly have different modules that are able to trigger internal initiation on their own (eg., Coldwell et al., 2000; Jopling et al., 2004; Stoneley et al., 1998). After establishment of *MTOR* IRES structure, it would be of full interest to address whether the entire structure is needed for ribosomal recruitment or if it has a modular composition. For that, deletion analysis of *MTOR* IRES must be performed, in which the different domains must be used individually and in combination to assay for *MTOR* IRES activity.

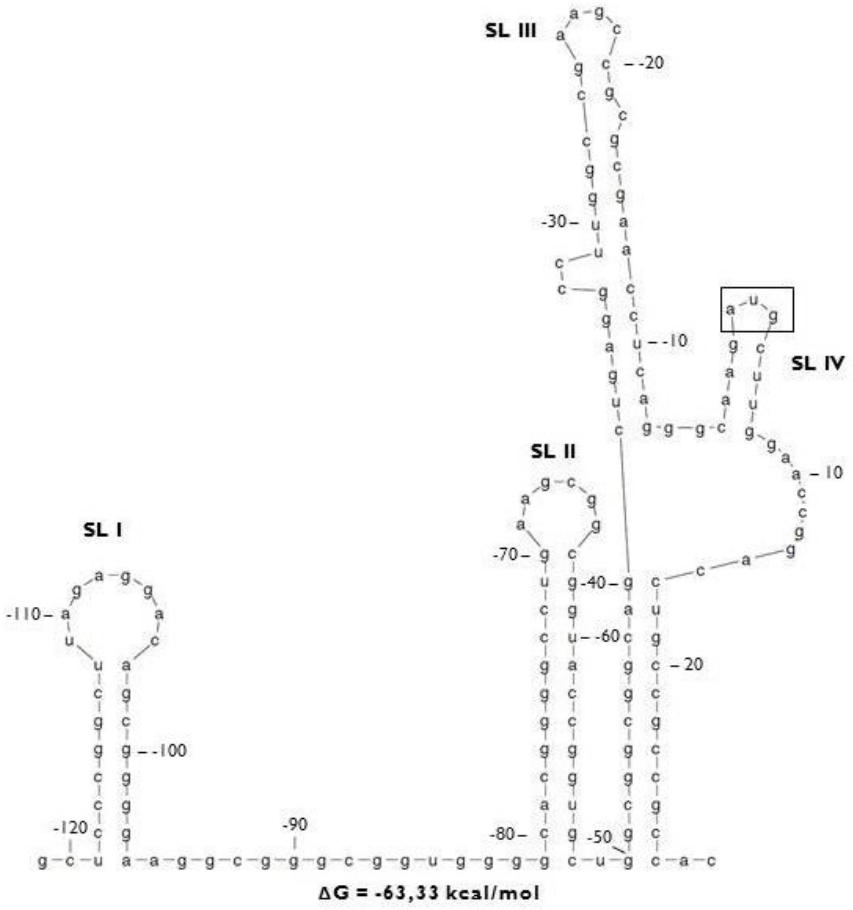


Figure VI.1. Representative RNA secondary structure of *MTOR* 5´UTR and part of its coding region, obtained by computer-assisted analysis. Sequences of different lengths of *MTOR* mRNA were submitted to M-Fold program (http://mfold.rna.albany.edu/?q=mfold) for prediction of secondary structures and a representative structure is presented. Putative stem-loop structures are shown, SL I from nucleotides (nts) -96 to -119, SL II from nts -52 to -81 and a composed structure (SL III and SL IV) from nts 27 to -50 (relatively to the AUG codon at position +1). Nucleotides position and the Gibb's free energy of the predicted secondary structure are indicated.

In line with this work, the secondary structure of the IRES for Δ160P53 has already been established (Candeias et al., unpublished data), although it was not addressed whether its ability to attract the translational apparatus relies on separate modules or on the overall structure. Accordingly, the same approach could be performed for the IRES governing Δ160P53 synthesis.

In the present study it was also demonstrated that the activity of the IRESs for MTOR and Δ160P53 is stimulated in conditions with associated increase in the

phosphorylation levels of EIF2α. Furthermore, it was also shown that IRES-driven translation of *MTOR* is enhanced upon MTORC1 inhibition. Those findings strongly suggest that the IRESs for MTOR and Δ160P53 are not only able to recruit the 40S ribosomal subunits independent of ternary complexes and that the former IRES element function in a manner that is independent of functional EIF4F complexes, but also that both possess competitive advantage for ribosomal recruitment in such conditions. It has been reported that other initiation factors can function as EIF2 substitutes in a way that they are also able to deliver Met-tRNAi to the 40S ribosomal subunit, such as EIF2A (Kim et al., 2011) EIF2D (Dmitriev et al., 2010), EIF5B (Pestova et al., 2008; Thakor and Holcik, 2011) or MCT1 in combination with DENR, which are homologous to N-terminal and C-terminal regions of EIF2D, respectively (Skabkin et al., 2010). Of particular interest is EIF2A, that stimulates Met-tRNAi binding to the 40S ribosomal subunit in a manner that is dependent on AUG (Zoll et al., 2002). EIF2A is involved in IRES-driven translation of HCV under stress conditions with increased EIF2α phosphorylation, through direct binding to a HCV IRES domain that also interacts with 40S ribosomal subunit. This proximity is probably responsible for the EIF2A-associated Met-tRNAi delivery to the 40S ribosomal P site (Kim et al., 2011). Similarly, *XIAP* IRES recruits the 40S ribosomal subunit in an EIF5B-dependent manner upon, augmented eIF2α phosphorylation (Thakor and Holcik, 2011). In addition, it has been demonstrated that the vast majority of IRES do not require the cap-binding protein EIF4E (reviewed in Hellen and Sarnow, 2001). In order to see whether the translational advantage conferred by the IRES elements for MTOR and Δ160P53 in the aforementioned stress conditions comes from the fact that their PICs are composed of initiation factors that are not used by the canonical cap-dependent mechanism or whether they bypass the requirement of EIFs that are required for the latter

mechanism, it would be of full interest to determine PIC composition of those IRESs elements, through mass spectrometry analysis of their native PICs.

The involvement of ITAFs constitutes an extra layer of regulation of IRES-dependent translation that might explain how different IRESs respond differently to the same stress conditions, such as occurs in etoposide-induced apoptosis in which the IRES within the *APAF1* transcript is active, whereas the IRES activity of *XIAP* is inhibited (Nevins et al., 2002; Warnakulasuriyarachchi et al., 2004); how some IRES are functional only in some physiological settings, such as the G2/M-dependent induction of the IRES for $CDK11^{p58}$ (Cornelis et al., 2000); how inhibitory conditions for cap-dependent translation are accompanied by stimulation of IRES-driven translation (reviewed in Spriggs et al., 2008) and the cell-type specificity of activation of some IRESs (eg., Candeias et al., 2006). It has been demonstrated that the nuclear-cytoplasmic shuttling of ITAFs might determine whether those accessory proteins are in close contact with their cognate IRESs elements in order to modulate their translational efficiency (reviewed in Komar and Hatzoglou, 2011). Accordingly, it would be of interest to determine the ITAFs involved in the IRESs governing MTOR and Δ160P53 expression, under unstressed and stressed conditions, and to test whether a subcellular redistribution is responsible for the stimulatory effect observed in translational adverse conditions. For that RNA electrophoretic mobility shift assays and RNA affinity pulldown assays combined with identification of each protein through mass-spectrometry and subsequent immunofluorescence of the correspondent ITAF could be performed.

As a next step, one could perform toeprinting analysis in order to identify the MTOR and Δ160P53 IRESs binding sequences for the components of the translational machinery.

The data presented here strongly suggest that the increase in EIF2α phosphorylation levels is accounting for the stimulatory effect on the activity of the IRESs for MTOR and Δ160P53, observed upon ER stress and also hypoxia, for the former IRES. In order to further confirm that EIF2α phosphorylation is responsible for this stimulation, one might assay for IRES activity upon ectopic expression of an unphophorylatable EIF2α protein. The same approach could be performed for 4EBP1 and S6K1, in order to check what MTORC1 targets are responsible for *MTOR* IRES stimulation upon rapamycin treatment. It is further demonstrated that a stimulation of *MTOR* IRES is observed in the anti-apoptotic outcome of ER stress, in which EIF2α is unphosphorylated or dephosphorylated, which prompted to the conclusion that another UPR event is stimulating *MTOR* IRES activity. In order to find what event is further inducing IRES-driven translation of *MTOR*, a first approach could be to impair each of the three UPR branches, namely by RNAi targeting IRE1, PERK and ATF6.

Here, it is proposed a model in which the *MTOR* IRES is fundamental for maintenance of MTOR protein levels and, thus, for preventing MTORC1 and MTORC2 signalling lost upon stress conditions with associated cap-dependent mRNA translation reduction. Since it was not addressed whether *MTOR* transcript is also translated by the cap-dependent mechanism, a first step to confirm our model would be to test whether *MTOR* transcript is also translated by the canonical cap-dependent mechanism. One approach to achieve that goal would be to address whether MTOR protein levels produced from a monocistronic transcript with or without a stable hairpin, in close proximity to the cap structure, are the same. If both mechanisms are present, one could validate our model by inhibiting *MTOR* IRES, namely through oligos targeting a sequence important for *MTOR* IRES structure or binding to the translational machinery, and analyze MTOR endogenous levels and the phosphorylation levels of

MTORC1 and MTORC2 targets upon and after stress conditions (namely, ER stress, hypoxia and rapamycin exposure). In hypoxia, evaluation of translational rate of *HIF1α* transcript upon functional or inhibited *MTOR* IRES would also give insights about the importance of this cis-regulatory element in MTOR-mediated increase in *HIF1α* mRNA translation (Bernardi et al., 2006; Laughner et al., 2001; Thomas et al., 2006).

It would be interesting to test whether the other MTORC1 and MTORC2 components are also IRES-driven translated and whether those cis-regulatory elements are also accounting for MTOR signalling maintenance upon adverse conditions.

In order to address whether the cap-dependent mechanism is also accounting for Δ160 protein expression, one could use the same approach as previously mentioned for the *MTOR* IRES.

The promising results of MTOR inhibitors in cell lines, mice and clinical trials in humans (reviewed in Pópulo et al., 2012), highlight the importance of MTOR inhibition in diseases with hyper-activated MTOR signaling, such as cancer. Inhibition of IRES-mediated translation of *MTOR* might constitute another therapeutic strategy to impair the adverse events arising from hyper-activation of MTOR signalling. By targeting the *MTOR* transcript itself, an inhibition of both MTORC1 and MTORC2 signalling pathways would be possible. Morpholinos are small (usually 25-nt) molecules that bind RNA sequences by base pairing and are used namely to impair translation. By a steric-blocking mechanism, morpholinos can be used to inhibit protein binding to a given RNA sequence (reviewed in Summerton, 1999). Accordingly, it is proposed that the development of a morpholino targeting the *MTOR* IRES domain(s) responsible for recruitment of the translational machinery would constitute a good way to inhibit MTOR expression and, concomitantly, MTORC1 and MTORC2 pathways. As

morpholinos also block scanning ribosomes, if *MTOR* is also translated by the cap-mediated mechanism, a dual inhibition would be achieved.

Since Δ160P53 is an oncogene protein (Candeias et al., unpublished data) a morpholino targeting the IRES for Δ160P53 would be of particular interest, as well.

VII. APPENDIX I

VII.1. INTRODUCTION

The APC plays a critical role in the Wnt signalling and is involved in cell adhesion, migration and differentiation, cell cycle control, transcription and apoptosis (Aoki and Taketo, 2007; Fearnhead et al., 2001). *APC* is a tumor suppressor gene and mutant forms of *APC* have been associated with CRC tumorigenesis. Germline mutations of this gene are associated with familial adenomatous polyposis (FAP), an autosomal dominant hereditary disease characterized by various (more than 100) adenomatous polyps in colon and rectum, some of which progress to cancer (Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991; Vasen et al., 2008). Furthermore, somatic mutations of *APC*, that occur in the majority of sporadic cases of CRC, are also found in small adenomas and microscopic dysplastic lesions (Jen et al., 1994; Mori et al., 1992; Powell et al., 1992; Smith et al., 1994a), suggesting its involvement in early stages of CRC development. A milder form of this disease, called attenuated familial adenomatous polyposis (AFAP) is characterized by fewer polyps with delayed onset and progression to CRC in comparison to FAP and is caused mostly by germline mutations in the *APC* gene (reviewed in Knudsen et al., 2003) . Mutations in the *APC* gene are generally frameshift or point mutations resulting in premature stop codons which lead to synthesis of truncated proteins, both in FAP and AFAP (Hernegger et al., 2002). The *APC* regions commonly mutated in AFAP patients are the 5´ end, 3´ end, exon 9, or intron 9 (reviewed in Knudsen et al., 2003). It has been suggested that CRC development at patients with AFAP require somatic mutations of both the wild-type and germline mutant *APC* alleles (Spirio et al., 1998; Su et al., 2000). An *APC* allele bearing a 5´ end mutation (codon 157 at exon 4) generates a shorter but functional

APC protein through IRES-driven internal initiation at codon 184 (Goss et al., 2002). Goss et al. suggested that this mechanism might explain the less severe phenotype observed in AFAP with 5´ end mutations (Goss et al., 2002). It has been reported that, regarding the 5´ end mutations of *APC*, mutation at codon 157 represents the most downstream mutation responsible for the AFAP phenotype (Spirio et al., 1993). A FAP phenotype is reported in patients with a nonsense mutation at codon 168 (Olschwang et al., 1993), such as with downstream mutations (Spirio et al., 1993), which might suggest a role for this DNA sequence. Accordingly, it would be interesting to study whether mutations affecting *APC* IRES (Goss et al., 2002) are responsible for this apparent functional boundary within the *APC* gene determining FAP *versus* AFAP phenotype. Thus, it was intended to deepen the study of *APC* IRES in terms, namely, of structural modifications of *APC* IRES element due to reported mutations.

Here, it is shown that the *APC* region from codon 157, with a nonsense mutation, to codon 184, has an acceptor splice site disabling the use of the dicistronic DNA reporter to assess for IRES activity. This splice acceptor site has an AG dinucleotide immediately before the excision point and is preceded by a polypirimidine rich tract. The sequence TGGGTAAGT within RLuc ORF function as a splice donor site when located at nt 242 and nt 884 originating a 799-bp and 157-bp intron, respectively.

VII.2. MATERIALS AND METHODS

VII.2.1. Plasmid constructs

The DNA sequence from codon 157 to 184 of *APC* was PCR amplified with primers #1 and #2 (Table VII.2.1). In parallel, the previously mentioned pR_Fhp-vector was amplified with primers #3 and #4. The respective fragments were subjected to SOEing PCR with primers #1 and #4 and the resultant PCR products were digested with XhoI and BsrGI and cloned into pR_Fhp- and phpR_F (Table VII.2.1). The resultant constructs were named pR_APC_Fhp- and phpR_APC_F, respectively. To generate the promoterless constructs, the pR_Fhp- and pR_APC_Fhp- constructs were digested with NheI/BglII, blunt-ended with Quick Blunting Kit (New England Biolabs) and re-ligated, originating the promoterless pR_Fhp- and pR_APC_Fhp- contructs, respectively. A splice donor site within Renilla ORF of the pR_APC_Fhp- plasmid was changed by site-directed mutagenesis, using primers #5 e #6.

Table VII.2.1. DNA oligonucleotides used in the current work.

Primer	Sequence
#1	CCGCTCGAGCGGTAGTATTACGCTCAACTTCAGAATC
#2	CATCGGCCATATCTGTTTGTAAGGAAAA
#3	TACAAACAGATATGGCCGATGCTAAGAACATT
#4	GTGAGAGAAGCGCACACAG
#5	CGCTCCAGATGAAATGGGCAAGTACATCAAGAGCTTC
#6	GAAGCTCTTGATGTACTTGCCCATTTCATCTGGAGCG
#7	GTCTCGAACTTAAGCTGCAG
#8	TTACACGGCGATCTTGCCG

VII.2.2. Cell culture and plasmid transfection

Sw480 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cells were kept at 37°C in a humidified atmosphere containing 5% CO_2. Transient transfection was performed using Lipofectamine 2000

(Invitrogen), according to manufacturer's instructions, in 35-mm plates and, then, harvested after 24h. To assay IRES activity, Sw480 were transfected with 1µg of each dicistronic plasmid alone or in combination with 1µg of a plasmid encoding β-galactosidase (β-gal).

VII.2.3. RNA isolation

Total RNA from transfected cells was prepared using Nucleospin RNA extraction II (Marcherey-Nagel) followed by treatment with RNase-free DNase I (Ambion) and purification by phenol:chloroform extraction.

VII.2.4. Reverse transcription-PCR

First strand cDNA synthesis from 1µg of total RNA was carried out using SuperScript II Reverse Transcriptase (Life Technologies) and oligod(T) primer, according to the manufacturer's standard protocol. cDNAs were PCR amplified using primers #7 and #8 (Figure VII.4), or #4 and #7 (Figure VII.5) (Table VII.2.1). To control for DNA contamination PCR reactions were also carried out without prior cDNA synthesis. Samples were analyzed by electrophoresis on 0.8% agarose gels. The resulting fragments were then gel-purified and sequenced.

VII.2.5. Luciferase assays

Lysis was performed in all cell lines with Passive Lysis Buffer (Promega) and then cells were subjected to a freeze-thaw cycle at -80°C to 37°C and centrifuged at maximum speed for 5 minutes. The cell lysates were used to determine luciferase activity with the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's standard protocol. Ten µL of cell lysate were assayed for FLuc and

RLuc enzymatic activities. Ratio is the unit of FLuc after normalized to RLuc, and each value was derived from three independent experiments.

VII.2.6. Statistical analysis

Data are presented as means ± standard deviation of at least three independent experiments. Test F was used for evaluation of variances equality. Student's two-tailed *t*-test was used for estimation of statistical significance. Significance for statistical analysis was defined as $p<0.05$.

VII.3. RESULTS AND DISCUSSION

VII.3.1. The region spanning codons 157 to 184 of *APC* allows Firefly luciferase activity in a dicistronic context

In order to confirm the existence of an IRES element at *APC* coding region between codons 157 (with a nonsense mutation) and 184, this region was cloned between RLuc and FLuc ORFs of the previously described dicistronic pR_Fhp- dicistronic plasmid, originating the pR_APC_Fhp- construct (Figure VII.1A). Sw480 cells were transiently transfected with the aforementioned constructs and luciferase activity was measured. FLuc activity of pR_APC_Fhp- construct was normalized to the activity units from RLuc expressed in the same mRNA. The subsequent ratio between FLuc and RLuc (F/R) was compared to that from the empty pR_Fhp- construct, arbitrarily set to 1 (Figure VII.1B). Results show that the putative *APC* IRES enhances FLuc activity 192-fold over background.

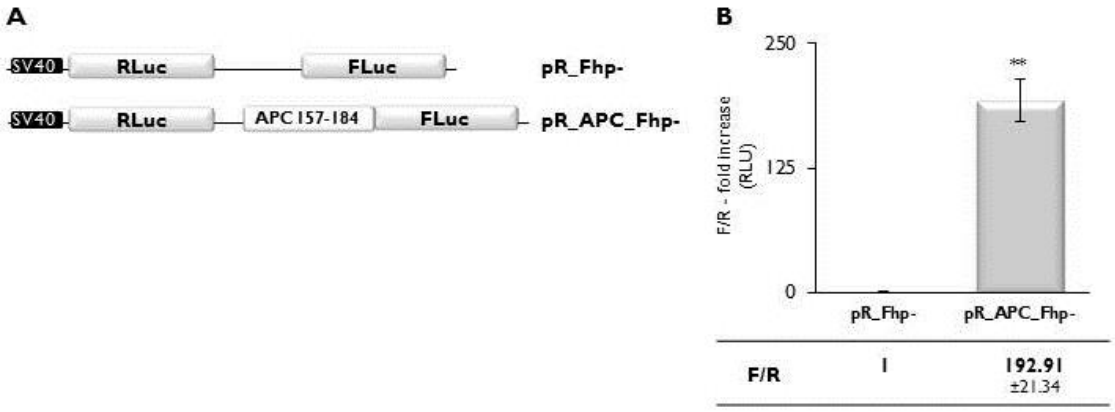


Figure VII.1. The region from codons 157 (with a nonsense mutation) to 184 of *APC* open reading frame (ORF) induces Firefly luciferase activity in a dicistronic context. (A) Scheme depicting the dicistronic constructs pR_Fhp- and pR_APC_Fhp- constructs. The *APC* open reading frame (ORF) spanning codons 157 (with a nonsense mutation) to 184 was cloned in the intercistronic space of the IRES-less plasmid vector (pR_Fhp-), originating the pR_APC_Fhp- plasmid. The dicistronic transcriptional units expressing Renilla luciferase (RLuc) ORF (RLuc box) and Firefly luciferase (FLuc) ORF (FLuc Box) are under the control of SV40 promoter. **(B)**

Relative enhancement of downstream reporter enzyme expression mediated by the putative *APC* IRES, comparing to that from the IRES-less plasmid vector. Sw480 cells were transiently transfected with the aforementioned plasmids and luciferase activity was measured 24 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between FLuc and RLuc, normalized to that of the empty pR_Fhp- construct, which was arbitrarily set to 1. Data are presented below each graph as the means ± standard deviation (SD) of at least 3 independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (**) $p<0.01$.

VII.3.2. The region spanning codons 157 to 184 of *APC* does not display cryptic promoter activity

Expression of a second cistron from a dicistronic DNA plasmid reporter vector might also be due to IRES-independent events, such as cryptic promoter activity or activation of splicing, producing aberrant monocistronic or dicistronic mRNAs encoding a functional protein (in this case, FLuc). In order to discard a false-positive result due to cryptic promoter activity of the *APC* region from codons 157 to 184, expression of FLuc derived from a promoterless plasmid was evaluated. For that, SV40 promoter was removed from the dicistronic DNA constructs pR_Fhp- and pR_APC_Fhp- (Figure VII.2A). Sw480 cells were transfected with the pR_Fhp- and pR_APC_Fhp- reporter plasmids with and without promoter along with a plasmid encoding β-galactosidase (β-gal). Renilla and Firefly luciferase activities were determined and normalized to the activity of β-gal. The subsequent ratios of RLuc to β-gal or FLuc to β-gal (R/β-gal or F/β-gal) were compared to those from the empty pR_Fhp- vector (Figure VII.2B), arbitrarily set to 1. Results showed that removal of SV40 reduced FLuc and RLuc activities of both plasmids to background levels, demonstrating that the observed FLuc expression driven by *APC* is not due to a cryptic promoter event. Interestingly, it was observed that insertion of the *APC* fragment upstream of FLuc, in the promoter-containing plasmid, also reduces RLuc activity when comparing to the pR_Fhp- empty vector. (Figure VII.2B).

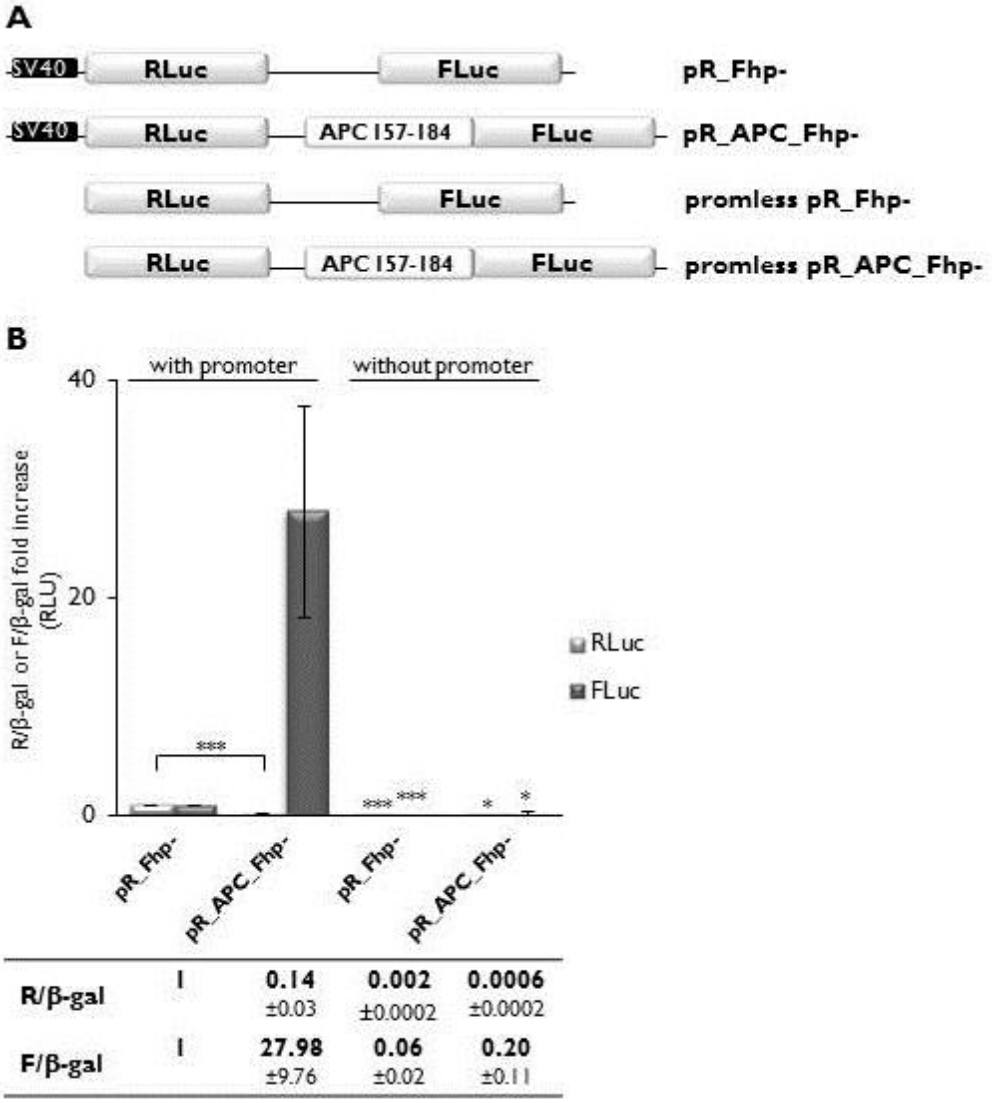


Figure VII.2. The region of *APC* open reading frame (ORF) between codons 157 (with a nonsense mutation) and 184 does not display cryptic promoter activity. (A) Scheme depicting the dicistronic constructs pR_Fhp- and pR_APC_Fhp- vectors with and without SV40 promoter, as in Figure VII.1. **(B)** Sw480 cells were transiently co-transfected with the dicistronic constructs with and without promoter (A) along with a plamid expressing β-galactosidase (β-gal). Luciferase activity was measured 24 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between Renilla luciferase (RLuc) and β-gal or Firefly luciferase (FLuc) and β-gal compared to that of the corresponding empty construct, which was arbitrarily set to 1. Data are presented below each graph as the means ± SD of three independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (∗) $p<0.05$; (∗∗∗) $p<0.001$.

In order to evaluate the cap independence of *APC*-driven FLuc expression, a stable hairpin was cloned upstream of RLuc of the pR_APC_Fhp- reporter, generating the phpR_APC_F construct (Figure VII.3A), and the R/β-gal or F/β-gal ratios were obtained as before and compared to those from the pR_APC_Fhp- vector (Figure VII.3A), arbitrarily set to 1. If FLuc expression derives solely from IRES-mediated translation, only RLuc activity will be reduced by this hairpin. Figure VII.3B shows that the hairpin exerts an inhibitory effect not only on RLuc activity but also on FLuc activity, suggesting that FLuc expression is somewhat dependent on RLuc.

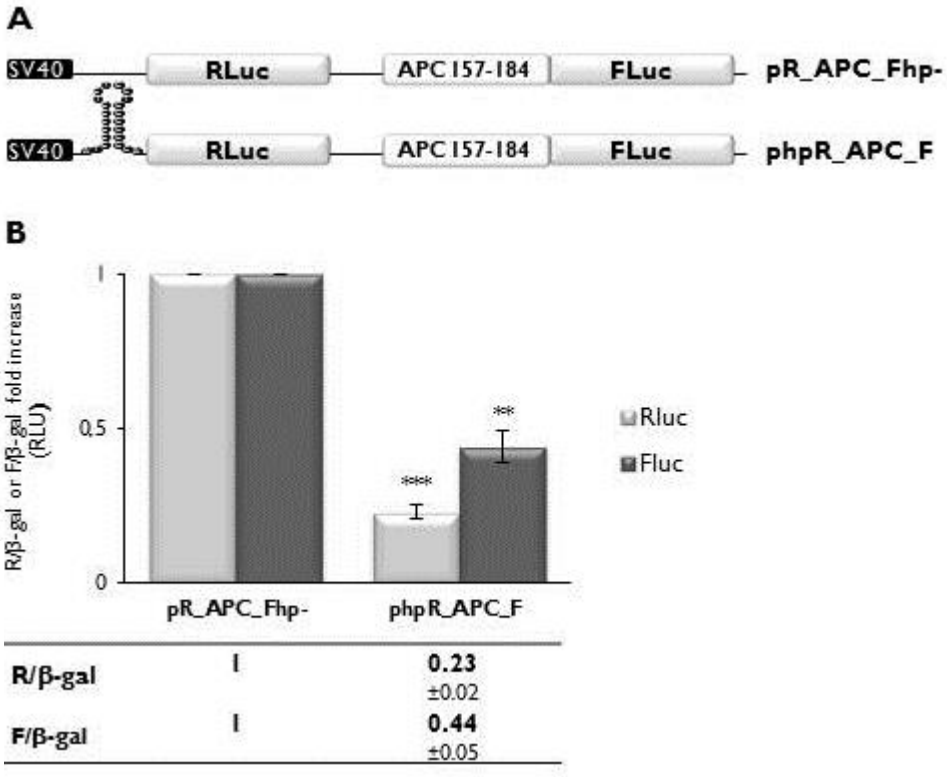


Figure VII.3. Firefly luciferase activity driven by the putative *APC* IRES is not independent of Renilla luciferase. (A) Scheme depicting the dicistronic DNA reporter pR_APC_Fhp- and phpR_APC_F constructs. A stable hairpin was cloned upstream of Renilla luciferase (RLuc) open reading frame (ORF) (RLuc box) of the pR_APC_Fhp- construct, originating phpR_APC_F. **(B)** Sw480 cells were transiently co-transfected with the dicistronic plasmids depicted in A along with a plasmid expressing β-galactosidase (β-gal). Luciferase activity was measured 24 hours post-transfection. The values (relative light units; RLU) are shown as the luminescence ratio between RLuc and β-gal or Firefly luciferase (FLuc) and β-gal compared to that of the pR_APC_Fhp- vector, which was arbitrarily set to 1. Data are presented below each

graph as the means ± SD of three independent experiments. Statistical analysis was performed using the Student's *t* test (unpaired, two-tailed); (**) $p<0.01$; (***) $p<0.001$.

VII.3.3. The dicistronic reporter carrying the putative *APC* IRES is subjected to cryptic splicing

It was wondered whether this dependence of *APC*-driven FLuc expression on RLuc was due to a splicing event. In order to check this hypothesis, the *APC* region under study in conjunction with RLuc ORF and part of FLuc coding region, was submitted to Splice View analysis, allowing a computer-assisted prediction of potential splice sites. As depicted in Table VII.3.1, two splice acceptor sites within *APC* were predicted. Both potential splice acceptor sites have an AG dinucleotide before the potential excision point, which might suggest that the GT-AG rule for splice junctions is obeyed (Mount, 1982).

Table VII.3.1. Four splice donor sites at the 3´ end of Renilla luciferase (RLuc) open reading frame (ORF) as well as two splice acceptor sites at *APC* ORF from codon 157 (with a nonsense mutation) to 184 were predicted. Potential splice sites were predicted by Splice View program (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html) through submission of the DNA sequence spanning Renilla luciferase (RLuc) open reading frame (ORF), the intercistronic region, the putative *APC* IRES sequence and the first 50 nucleotides (nts) of FLuc ORF from the pR_APC_Fhp- (Figure VII.1A) construct. The nt numbering is referred to the translation initiation site of RLuc and the 5´ end of the putative *APC* IRES, for splice donor and acceptor sites, respectively. *PDS - Predicted splice Donor Site; PAS - Predicted splice Acceptor Site.*

Predicted splice Donor Sites upstream of the putative *APC* IRES		
Identification	**Exon/Intron**	**Localization**
PDS#1	TGG/GTAAGT	RLuc ORF nt 242 – 250
PDS#2	GAG/GTTAGA	RLuc ORF nt 631 – 639
PDS#3	AAG/GTGAAG	RLuc ORF nt 838 – 846
PDS#4	TGG/GTAAGT	RLuc ORF nt 884 – 892
Predicted splice Acceptor Sites at the putative *APC* IRES		
Identification	**Intron/Exon**	**Localization**
PAS#1	CAACTTCAG/AATC	Nt 13 – 25
PAS#2	TACAAACAG/ATAT	Nt 71 – 83

To confirm this prediction, the integrity of the RNA expressed from pR_APC_Fhp- was checked through RT-PCR, and the generated PCR fragments were sequenced. As shown in Figure VII.4A, a single fragment was generated by PCR amplification with one pair of oligonucleotide primers spanning the entire transcript until FLuc codon stop. However, sequencing of the generating PCR fragment revealed that the region from nt 887 of RLuc coding region to nt 79 of the putative *APC* IRES was deleted (Figure VII.4B). The nt 79 is the "G" from the dinucleotide AG of PAS#2 (Table VII.3.1). Splice View also predicted several potential splice donor sites at RLuc ORF 3´end (Table VII.3.1). Of interest, the excision site for PDS#4 is at nt 886. This potential donor site almost perfectly matches the consensus sequence: AG|GTRAGT for splice donor sites (Senapathy et al., 1990). Furthermore, a string of nine pyrimidines is found 6 nts upstream of the PAS#2 excision point. It is known that the polypyrimidine tract adjacent to the excision point of a splice acceptor site is an important binding site for splice factors (Reed, 1989). Accordingly, this result strongly suggests that the RNA expressed from the pR_APC_Fhp- reporter was subjected to cryptic splicing, generating an aberrant monocistronic transcript containing RLuc ORF with a 3´deletion of 50-nts followed by two 3´ end nts from the *APC* sequence under study, and the entire FLuc ORF, which is in-frame with RLuc ORF. This mRNA encodes a RLuc-FLuc fusion protein, in which the sixteen C-terminal amino acids of RLuc are lacking, the most C-terminal glycine residue is substituted by aspartate and the FLuc protein is complete, explaining the RLuc expression-dependency on FLuc activity and the decrease of RLuc activity promoted by the *APC* sequence.

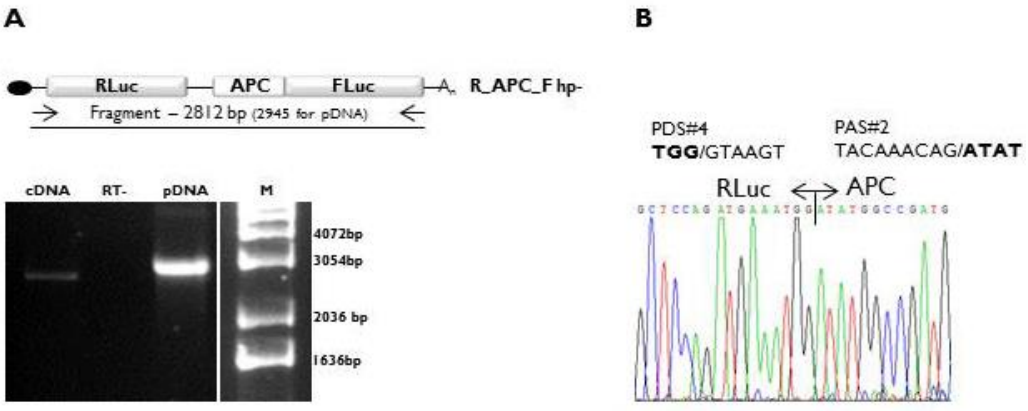


Figure VII.4. The dicistronic reporter carrying the putative *APC* IRES is subjected to cryptic splicing resulting in excision of a 157-nt intron. Sw480 were transiently transfected with the dicistronic pR_APC_Fhp- construct. Twenty-four hours later, RNA was extracted and RT-PCR was performed with one pair of oligonucleotide primers (represented by arrows), originating a fragment spanning the entire transcript until the 3´ end of Firefly luciferase (FLuc) cistron. **(A)** Ethidium bromide-stained agarose gel showing RT-PCR products. A single PCR fragment is observed. *cDNA: presence of cDNA; -RT: absence of cDNA; pDNA: pR_APC_Fhp- vector, containing a 133-bp chimeric intron upstream of Renilla luciferase (RLuc); M: molecular weight marker (NZY Ladder III).* **(B)** Sequencing chromatogram demonstrating deletion of the DNA sequence between nt 887 from RLuc open reading frame (ORF) and nt 79 from the putative *APC* IRES. *PDS - Predicted splice Donor Site; PAS - Predicted splice Acceptor Site.*

In order to prevent the occurrence of cryptic splicing, the PDS#4 (Table VII.3.1) from pR_APC_Fhp- plasmid was mutated and a similar approach as before. As shown in Figure VII.5A, PCR amplification using a pair of primers spanning the RLuc coding region and part of FLuc ORF generated three fragments. By sequencing the generated fragments, it was realized that fragment A did not show any deletion (data not shown), in opposition to fragment C which lacked a 799-bp sequence from nt 245 of RLuc ORF to nt 79 of the putative *APC* IRES (Figure VII.5B). The fact that the excision point of PDS#1 (Table VII.3.1) is at nt 244 strongly suggests that this deletion corresponds to removal of a 799-bp cryptic intron. The resultant spliced transcript is similar to the observed previously, except for the RLuc ORF 3´ end deletion that is, in this case, of 692 nts. Fragment B showed a mixture of DNA sequences downstream of nt 245 of RLuc ORF (data not shown), suggesting the occurrence of other cryptic splicing events

at this region. Together these data demonstrate that the *APC* fragment tested for IRES activity induces alternative splicing in the dicistronic reporter used, disabling the analysis of IRES activity.

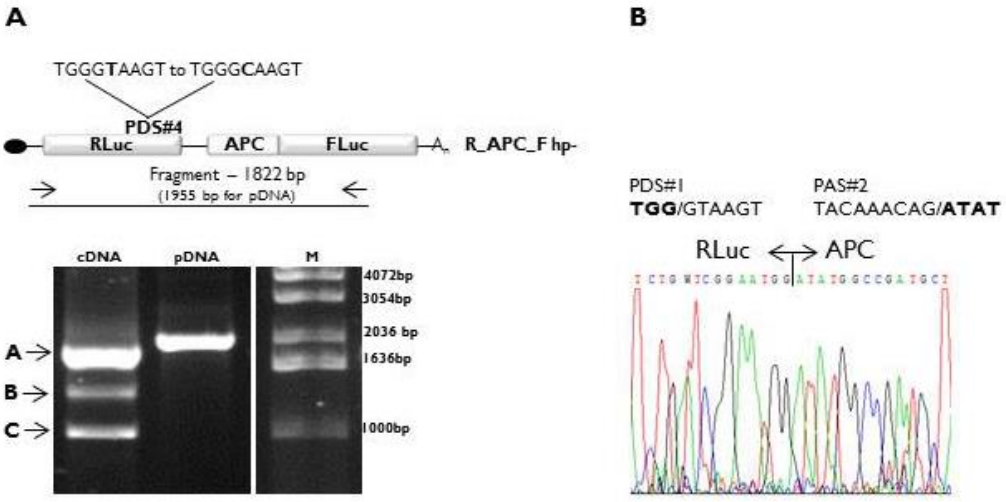


Figure VII.5. The dicistronic DNA reporter construct carrying the putative *APC* IRES and harboring a point mutation at PDS#4 expresses alternatively spliced mRNAs. A silent mutation was introduced at PDS#4 of the dicistronic pR_APC_Fhp- construct, by site-directed mutagenesis. Sw480 cells were transiently transfected with the generated plasmid vector. Twenty-four hours later, RNA was extracted and RT-PCR was performed with one pair of oligonucleotide primers (represented by arrows). **(A)** Ethidium bromide-stained agarose gel showing RT-PCR products. Besides the correct 1822-bp fragment (fragment A), two additional fragments (B and C) are observed, indicating for the occurrence of alternative splicing. *cDNA: presence of cDNA; pDNA: pR_APC_Fhp- vector, containing a 133-bp chimeric intron upstream of Renilla luciferase (RLuc); M: molecular weight marker (NZY Ladder III).* **(B)** Sequencing chromatogram of PCR fragment C demonstrating deletion of the DNA sequence between nt 245 from RLuc open reading frame (ORF) and nt 79 from the putative *APC* IRES. *PDS - Predicted splice Donor Site; PAS - Predicted splice Acceptor Site.*

VIII. BIBLIOGRAPHY

Abraham, N., Stojdl, D.F., Duncan, P.I., Méthot, N., Ishii, T., Dubé, M., Vanderhyden, B.C., Atkins, H.L., Gray, D.A., McBurney, M.W., et al. (1999). Characterization of transgenic mice with targeted disruption of the catalytic domain of the double-stranded RNA-dependent protein kinase, PKR. J. Biol. Chem. *274*, 5953–5962.

Abramson, R.D., Dever, T.E., and Merrick, W.C. (1988). Biochemical evidence supporting a mechanism for cap-independent and internal initiation of eukaryotic mRNA. J. Biol. Chem. *263*, 6016–6019.

Acker, M.G., Shin, B.-S., Nanda, J.S., Saini, A.K., Dever, T.E., and Lorsch, J.R. (2009). Kinetic analysis of late steps of eukaryotic translation initiation. J. Mol. Biol. *385*, 491–506.

Ahrendt, S.A., Hu, Y., Buta, M., McDermott, M.P., Benoit, N., Yang, S.C., Wu, L., and Sidransky, D. (2003). p53 Mutations and Survival in Stage I Non-Small-Cell Lung Cancer: Results of a Prospective Study. JNCI J. Natl. Cancer Inst. *95*, 961–970.

Aitken, C.E. (2012). A mechanistic overview of translation initiation in eukaryotes : Nature Structural & Molecular Biology : Nature Publishing Group.

Al-Fageeh, M.B., and Smales, C.M. (2009). Cold-inducible RNA binding protein (CIRP) expression is modulated by alternative mRNAs. RNA *15*, 1164–1176.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. *15*, 6541–6551.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Curr. Biol. *7*, 261–269.

Ali, I.K., McKendrick, L., Morley, S.J., and Jackson, R.J. (2001). Activity of the hepatitis A virus IRES requires association between the cap-binding translation initiation factor (eIF4E) and eIF4G. Journal of Virology *75*, 7854.

Allagnat, F., Cunha, D., Moore, F., Vanderwinden, J.M., Eizirik, D.L., and Cardozo, A.K. (2011). Mcl-1 downregulation by pro-inflammatory cytokines and palmitate is an early event contributing to β-cell apoptosis. Cell Death Differ. *18*, 328–337.

Allam, H., and Ali, N. (2009). Initiation Factor eIF2-independent Mode of c-Src mRNA Translation Occurs via an Internal Ribosome Entry Site. J. Biol. Chem. *285*, 5713–5725.

Amaravadi, R.K., Yu, D., Lum, J.J., Bui, T., Christophorou, M.A., Evan, G.I., Thomas-Tikhonenko, A., and Thompson, C.B. (2007). Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J. Clin. Invest. *117*, 326–336.

Anderson, D.D., and Stover, P.J. (2009). SHMT1 and SHMT2 Are Functionally Redundant in Nuclear De novo Thymidylate Biosynthesis. PLoS ONE *4*, e5839.

Andreev, D.E., Fernandez-Miragall, O., Ramajo, J., Dmitriev, S.E., Terenin, I.M., Martinez-Salas, E., and Shatsky, I.N. (2007). Differential factor requirement to assemble translation initiation complexes at the alternative start codons of foot-and-mouth disease virus RNA. RNA *13*, 1366–1374.

Aoki, K., and Taketo, M.M. (2007). Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. J. Cell Sci. *120*, 3327–3335.

Aoubala, M., Murray-Zmijewski, F., Khoury, M.P., Fernandes, K., Perrier, S., Bernard, H., Prats, A.-C., Lane, D.P., and Bourdon, J.-C. (2011). p53 directly transactivates

Δ133p53α, regulating cell fate outcome in response to DNA damage. Cell Death Differ. *18*, 248–258.

Apcher, S., Komarova, A., Daskalogianni, C., Yin, Y., Malbert-Colas, L., and Fahraeus, R. (2008). mRNA Translation Regulation by the Gly-Ala Repeat of Epstein-Barr Virus Nuclear Antigen 1. J. Virol. *83*, 1289–1298.

Appenzeller-Herzog, C., and Hall, M.N. (2012). Bidirectional crosstalk between endoplasmic reticulum stress and MTOR signaling. Trends Cell Biol. *22*, 274–282.

Arif, A., Jia, J., Mukhopadhyay, R., Willard, B., Kinter, M., and Fox, P.L. (2009). Two-Site Phosphorylation of EPRS Coordinates Multimodal Regulation of Noncanonical Translational Control Activity. Mol. Cell *35*, 164–180.

Arita, M., Zhong, X., Min, Z., Hemmi, H., and Shimatake, H. (2003). Multiple sites required for expression in 5′-flanking region of the hMLH1 gene. Gene *306*, 57–65.

Armengol, G., Rojo, F., Castellví, J., Iglesias, C., Cuatrecasas, M., Pons, B., Baselga, J., and Ramón y Cajal, S. (2007). 4E-binding protein 1: a key molecular "funnel factor" in human cancer with clinical implications. Cancer Res. *67*, 7551–7555.

Arsham, A.M. (2003). A Novel Hypoxia-inducible Factor-independent Hypoxic Response Regulating Mammalian Target of Rapamycin and Its Targets. J. Biol. Chem. *278*, 29655–29660.

Asano, K., Shalev, A., Phan, L., Nielsen, K., Clayton, J., Valasek, L., Donahue, T.F., and Hinnebusch, A.G. (2001). Multiple roles for the C-terminal domain of eIF5 in translation initiation complex assembly and GTPase activation. EMBO J *20*, 2326–2337.

Avdulov, S., Li, S., Michalek, V., Burrichter, D., Peterson, M., Perlman, D.M., Manivel, J.C., Sonenberg, N., Yee, D., Bitterman, P.B., et al. (2004). Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells. Cancer Cell *5*, 553–563.

Avery-Kiejda, K.A., Zhang, X.D., Adams, L.J., Scott, R.J., Vojtesek, B., Lane, D.P., and Hersey, P. (2008). Small Molecular Weight Variants of p53 Are Expressed in Human Melanoma Cells and Are Induced by the DNA-Damaging Agent Cisplatin. Clinical Cancer Research *14*, 1659–1668.

Babendure, J.R., Babendure, J.L., Ding, J.-H., and Tsien, R.Y. (2006). Control of mammalian translation by mRNA structure near caps. RNA *12*, 851–861.

Bachar, E., Ariav, Y., Ketzinel-Gilad, M., Cerasi, E., Kaiser, N., and Leibowitz, G. (2009). Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of MTORC1. PloS One *4*, e4954.

Baird, S.D. (2006). Searching for IRES. RNA *12*, 1755–1785.

Bar-Peled, L., Schweitzer, L.D., Zoncu, R., and Sabatini, D.M. (2012). Ragulator Is a GEF for the Rag GTPases that Signal Amino Acid Levels to MTORC1. Cell *150*, 1196–1208.

Basu, A., Das, P., Chaudhuri, S., Bevilacqua, E., Andrews, J., Barik, S., Hatzoglou, M., Komar, A.A., and Mazumder, B. (2011). Requirement of rRNA Methylation for 80S Ribosome Assembly on a Cohort of Cellular Internal Ribosome Entry Sites. Molecular and Cellular Biology *31*, 4482–4499.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., et al. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science *244*, 217–221.

Bayle, J.H., Elenbaas, B., and Levine, A.J. (1995). The carboxyl-terminal domain of the p53 protein regulates sequence-specific DNA binding through its nonspecific

nucleic acid-binding activity. Proceedings of the National Academy of Sciences *92*, 5729–5733.

Bellacosa, A., Chan, T.O., Ahmed, N.N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998). Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene *17*, 313–325.

Bellodi, C., Kopmar, N., and Ruggero, D. (2010a). Deregulation of oncogene-induced senescence and p53 translational control in X-linked dyskeratosis congenita. EMBO J. *29*, 1865–1876.

Bellodi, C., Krasnykh, O., Haynes, N., Theodoropoulou, M., Peng, G., Montanaro, L., and Ruggero, D. (2010b). Loss of function of the tumor suppressor DKC1 perturbs p27 translation control and contributes to pituitary tumorigenesis. Cancer Res. *70*, 6026–6035.

Belsham, G.J. (1992). Dual initiation sites of protein synthesis on foot-and-mouth disease virus RNA are selected following internal entry and scanning of ribosomes in vivo. EMBO J. *11*, 1105.

Belsham, G.J., and R. J. Jackson. 2000. Translation initiation on picornavirus RNA, p. 869-900. *In* N. Sonenberg, J. W. B. Hershey, and M. B. Mathews (ed.), Translational control of gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Bensaad, K., and Vousden, K.H. (2007). p53: new roles in metabolism. Trends Cell Biol. *17*, 286–291.

Beretta, L., Gingras, A.C., Svitkin, Y.V., Hall, M.N., and Sonenberg, N. (1996). Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J. *15*, 658.

Bernard, H., Garmy-Susini, B., Ainaoui, N., Van Den Berghe, L., Peurichard, A., Javerzat, S., Bikfalvi, A., Lane, D.P., Bourdon, J.C., and Prats, A.-C. (2013). The p53 isoform, Δ133p53α, stimulates angiogenesis and tumour progression. Oncogene *32*, 2150–2160.

Bernardi, R., Guernah, I., Jin, D., Grisendi, S., Alimonti, A., Teruya-Feldstein, J., Cordon-Cardo, C., Celeste Simon, M., Rafii, S., and Pandolfi, P.P. (2006). PML inhibits HIF-1α translation and neoangiogenesis through repression of MTOR. Nature *442*, 779–785.

Bertout, J.A., Patel, S.A., and Simon, M.C. (2008). The impact of O2 availability on human cancer. Nat. Rev. Cancer *8*, 967–975.

Bi, M., Naczki, C., Koritzinsky, M., Fels, D., Blais, J., Hu, N., Harding, H., Novoa, I., Varia, M., and Raleigh, J. (2005). ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. The EMBO Journal *24*, 3470–3481.

Blais, J.D., Filipenko, V., Bi, M., Harding, H.P., Ron, D., Koumenis, C., Wouters, B.G., and Bell, J.C. (2004). Activating Transcription Factor 4 Is Translationally Regulated by Hypoxic Stress. Mol. Cell. Biol. *24*, 7469–7482.

Błaszczyk, L., and Ciesiołka, J. (2011). Secondary structure and the role in translation initiation of the 5'-terminal region of p53 mRNA. Biochemistry (Mosc.) *50*, 7080–7092.

Blommaart, E.F., Luiken, J.J., Blommaart, P.J., van Woerkom, G.M., and Meijer, A.J. (1995). Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. J. Biol. Chem. *270*, 2320–2326.

Bommer, G.T., Gerin, I., Feng, Y., Kaczorowski, A.J., Kuick, R., Love, R.E., Zhai, Y., Giordano, T.J., Qin, Z.S., Moore, B.B., et al. (2007). p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr. Biol. CB *17*, 1298–1307.

Bommiasamy, H., Back, S.H., Fagone, P., Lee, K., Meshinchi, S., Vink, E., Sriburi, R., Frank, M., Jackowski, S., Kaufman, R.J., et al. (2009). ATF6α induces XBP1-independent expansion of the endoplasmic reticulum. J. Cell Sci. *122*, 1626–1636.

Bourdon, J.-C., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, D.P., Saville, M.K., and Lane, D.P. (2005). p53 isoforms can regulate p53 transcriptional activity. Genes Dev. *19*, 2122–2137.

Bourougaa, K., Naski, N., Boularan, C., Mlynarczyk, C., Candeias, M.M., Marullo, S., and Fåhraeus, R. (2010). Endoplasmic Reticulum Stress Induces G2 Cell-Cycle Arrest via mRNA Translation of the p53 Isoform p53/47. Mol. Cell *38*, 78–88.

Boutsma, E., Noback, S., and van Lohuizen, M. (2008). The Polycomb protein and E3 ubiquitin ligase Ring1B harbors an IRES in its highly conserved 5' UTR. PLoS ONE *3*, e2322.

Brandish, P.E., Kimura, K.I., Inukai, M., Southgate, R., Lonsdale, J.T., and Bugg, T.D. (1996). Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from Escherichia coli. Antimicrob. Agents Chemother. *40*, 1640–1644.

Braunstein, S., Karpisheva, K., Pola, C., Goldberg, J., Hochman, T., Yee, H., Cangiarella, J., Arju, R., Formenti, S.C., and Schneider, R.J. (2007). A Hypoxia-Controlled Cap-Dependent to Cap-Independent Translation Switch in Breast Cancer. Molecular Cell *28*, 501–512.

Brown, C.M., Stockwell, P.A., Trotman, C.N.A., and Tate, W.P. (1990). Sequence analysis suggests that tetra-nucleotides signal the termination of protein synthesis in eukaryotes. Nucleic Acids Res. *18*, 6339–6345.

Brown, E.J., Albers, M.W., Bum Shin, T., ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature *369*, 756–758.

Brown, J.P., Wei, W., and Sedivy, J.M. (1997). Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science *277*, 831–834.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W., and Kaelin, W.G., Jr (2004). Regulation of MTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev. *18*, 2893–2904.

Brunn, G.J., Hudson, C.C., Sekulić, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C., and Abraham, R.T. (1997). Phosphorylation of the Translational Repressor PHAS-I by the Mammalian Target of Rapamycin. Science *277*, 99–101.

Budanov, A.V., and Karin, M. (2008). p53 Target Genes Sestrin1 and Sestrin2 Connect Genotoxic Stress and MTOR Signaling. Cell *134*, 451–460.

Burnett, P.E., Barrow, R.K., Cohen, N.A., Snyder, S.H., and Sabatini, D.M. (1998). RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc. Natl. Acad. Sci. U. S. A. *95*, 1432–1437.

Bushell, M., McKendrick, L., Jänicke, R.U., Clemens, M.J., and Morley, S.J. (1999). Caspase-3 is necessary and sufficient for cleavage of protein synthesis eukaryotic initiation factor 4G during apoptosis. FEBS Lett. *451*, 332–336.

Bushell, M., Stoneley, M., Kong, Y.W., Hamilton, T.L., Spriggs, K.A., Dobbyn, H.C., Qin, X., Sarnow, P., and Willis, A.E. (2006). Polypyrimidine Tract Binding Protein Regulates IRES-Mediated Gene Expression during Apoptosis. Mol. Cell *23*, 401–412.

Calvo, S.E., Pagliarini, D.J., and Mootha, V.K. (2009). Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. Proc. Natl. Acad. Sci. U.S.A. *106*, 7507–7512.

Cam, H., and Houghton, P.J. (2011). Regulation of mammalian target of rapamycin complex 1 (MTORC1) by hypoxia: causes and consequences. Target. Oncol. *6*, 95–102.

Cam, H., Easton, J.B., High, A., and Houghton, P.J. (2010). MTORC1 Signaling under Hypoxic Conditions Is Controlled by ATM-Dependent Phosphorylation of HIF-1α. Mol. Cell *40*, 509–520.

Cammas, A., Pileur, F., Bonnal, S., Lewis, S.M., Lévêque, N., Holcik, M., and Vagner, S. (2007). Cytoplasmic Relocalization of Heterogeneous Nuclear Ribonucleoprotein A1 Controls Translation Initiation of Specific mRNAs. Mol. Biol. Cell *18*, 5048–5059.

Candau, R., Scolnick, D.M., Darpino, P., Ying, C.Y., Halazonetis, T.D., and Berger, S.L. (1997). Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity. Oncogene *15*, 807–816.

Candeias, M.M., Powell, D.J., Roubalova, E., Apcher, S., Bourougaa, K., Vojtesek, B., Bruzzoni-Giovanelli, H., and Faahraeus, R. (2006). Expression of p53 and p53/47 are controlled by alternative mechanisms of messenger RNA translation initiation. Oncogene *25*, 6936–6947.

Candeias, M.M., Malbert-Colas, L., Powell, D.J., Daskalogianni, C., Maslon, M.M., Naski, N., Bourougaa, K., Calvo, F., and Fahraeus, R. (2008). p53 mRNA controls p53 activity by managing Mdm2 functions. Nat Cell Biol *10*, 1098–1105.

Carriere, A., Ray, H., Blenis, J., and Roux, P.P. (2008). The RSK factors of activating the Ras/MAPK signaling cascade. Front. Biosci. J. Virtual Libr. *13*, 4258–4275.

Cassan, M., and Rousset, J.-P. (2001). UAG readthrough in mammalian cells: effect of upstream and downstream stop codon contexts reveal different signals. BMC Mol. Biol. *2*, 3.

Castro, A.F., Rebhun, J.F., Clark, G.J., and Quilliam, L.A. (2003). Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. J. Biol. Chem. *278*, 32493–32496.

Chan, C.-P., Kok, K.-H., Tang, H.-M.V., Wong, C.-M., and Jin, D.-Y. (2013). Internal ribosome entry site-mediated translational regulation of ATF4 splice variant in mammalian unfolded protein response. Biochim. Biophys. Acta BBA-Mol. Cell Res.

Chang, C.-J., Chao, C.-H., Xia, W., Yang, J.-Y., Xiong, Y., Li, C.-W., Yu, W.-H., Rehman, S.K., Hsu, J.L., Lee, H.-H., et al. (2011). p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. Nat. Cell Biol. *13*, 317–323.

Chang, J.H., Cho, Y.H., Sohn, S.Y., Choi, J.M., Kim, A., Kim, Y.C., Jang, S.K., and Cho, Y. (2009). Crystal structure of the eIF4A–PDCD4 complex. Proc. Natl. Acad. Sci. *106*, 3148–3153.

Chang, T.-C., Wentzel, E.A., Kent, O.A., Ramachandran, K., Mullendore, M., Lee, K.H., Feldmann, G., Yamakuchi, M., Ferlito, M., Lowenstein, C.J., et al. (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol. Cell *26*, 745–752.

Chappell, S.A., Edelman, G.M., and Mauro, V.P. (2004). Biochemical and functional analysis of a 9-nt RNA sequence that affects translation efficiency in eukaryotic cells. Proc. Natl. Acad. Sci. U. S. A. *101*, 9590.

Chappell, S.A., LeQuesne, J.P., Paulin, F.E., deSchoolmeester, M.L., Stoneley, M., Soutar, R.L., Ralston, S.H., Helfrich, M.H., and Willis, A.E. (2000). A mutation in the c-

myc-IRES leads to enhanced internal ribosome entry in multiple myeloma: a novel mechanism of oncogene de-regulation. Oncogene *19*, 4437–4440.

Chaudhuri, S., Vyas, K., Kapasi, P., Komar, A.A., Dinman, J.D., Barik, S., and Mazumder, B. (2007). Human ribosomal protein L13a is dispensable for canonical ribosome function but indispensable for efficient rRNA methylation. RNA *13*, 2224–2237.

Chauvin, C., Koka, V., Nouschi, A., Mieulet, V., Hoareau-Aveilla, C., Dreazen, A., Cagnard, N., Carpentier, W., Kiss, T., Meyuhas, O., et al. (2013). Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program. Oncogene.

Chen, J., Guo, K., and Kastan, M.B. (2012). Interactions of nucleolin and ribosomal protein L26 (RPL26) in translational control of human p53 mRNA. J. Biol. Chem. *287*, 16467–16476.

Chen, C., and Sarnow, P. (1995). Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. Science *268*, 415–417.

Chen, J.-J., and Perrine, S. (2013). Stressing HbF synthesis: role of translation? Blood *122*, 467–468.

Chen, J., and Kastan, M.B. (2010). 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes Dev. *24*, 2146–2156.

Chen, J., and Sadowski, I. (2005). Identification of the mismatch repair genes PMS2 and MLH1 as p53 target genes by using serial analysis of binding elements. Proc. Natl. Acad. Sci. U. S. A. *102*, 4813–4818.

Chen, J., Marechal, V., and Levine, A.J. (1993). Mapping of the p53 and mdm-2 interaction domains. Mol. Cell. Biol. *13*, 4107–4114.

Chen, J., Ng, S.M., Chang, C., Zhang, Z., Bourdon, J.-C., Lane, D.P., and Peng, J. (2009). p53 isoform Δ113p53 is a p53 target gene that antagonizes p53 apoptotic activity via BclxL activation in zebrafish. Genes Dev. *23*, 278–290.

Chen, S.-J., Lin, G., Chang, K.-J., Yeh, L.-S., and Wang, C.-C. (2008). Translational Efficiency of a Non-AUG Initiation Codon Is Significantly Affected by Its Sequence Context in Yeast. J. Biol. Chem. *283*, 3173–3180.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.-K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., et al. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725–730.

Chiu, S.-Y., Lejeune, F., Ranganathan, A.C., and Maquat, L.E. (2004). The pioneer translation initiation complex is functionally distinct from but structurally overlaps with the steady-state translation initiation complex. Genes Dev. *18*, 745–754.

Cho, S., Kim, J.H., Back, S.H., and Jang, S.K. (2005). Polypyrimidine Tract-Binding Protein Enhances the Internal Ribosomal Entry Site-Dependent Translation of p27Kip1 mRNA and Modulates Transition from G1 to S Phase. Mol. Cell. Biol. *25*, 1283–1297.

Choi, J., Chen, J., Schreiber, S.L., and Clardy, J. (1996). Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. Science *273*, 239–242.

Choo, A.Y., Yoon, S.O., Kim, S.G., Roux, P.P., and Blenis, J. (2008). Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. Proc. Natl. Acad. Sci. *105*, 17414.

Clemens, M.J., Bushell, M., Jeffrey, I.W., Pain, V.M., and Morley, S.J. (2000). Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. Cell Death Differ. *7*, 603–615.

Clore, G.M., Ernst, J., Clubb, R., Omichinski, J.G., Kennedy, W.M., Sakaguchi, K., Appella, E., and Gronenborn, A.M. (1995). Refined solution structure of the oligomerization domain of the tumour suppressor p53. Nat. Struct. Biol. *2*, 321–333.

Cobbold, L.C., Wilson, L.A., Sawicka, K., King, H.A., Kondrashov, A.V., Spriggs, K.A., Bushell, M., and Willis, A.E. (2010). Upregulated c-myc expression in multiple myeloma by internal ribosome entry results from increased interactions with and expression of PTB-1 and YB-1. Oncogene.

Coldwell, M.J., Mitchell, S.A., Stoneley, M., MacFarlane, M., and Willis, A.E. (2000). Initiation of APAF1 translation by internal ribosome entry. Oncogene *19*, 899–905.

Conn, C.S., and Qian, S.-B. (2013). Nutrient signaling in protein homeostasis: an increase in quantity at the expense of quality. Sci. Signal. *6*, ra24.

Cooper GM. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Translation of mRNA. Available from: http://www.ncbi.nlm.nih.gov/books/NBK9849/

Cornelis, S., Bruynooghe, Y., Denecker, G., Van Huffel, S., Tinton, S., and Beyaert, R. (2000). Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Mol. Cell *5*, 597–605.

Costantino, D.A., Pfingsten, J.S., Rambo, R.P., and Kieft, J.S. (2008). tRNA-mRNA mimicry drives translation initiation from a viral IRES. Nat Struct Mol Biol *15*, 57–64.

Courtois, S., Verhaegh, G., North, S., Luciani, M.-G., Lassus, P., Hibner, U., Oren, M., and Hainaut, P. (2002). DeltaN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. Oncogene *21*, 6722–6728.

Creancier, L., Mercier, P., Prats, A.-C., and Morello, D. (2001). c-myc Internal Ribosome Entry Site Activity Is Developmentally Controlled and Subjected to a Strong Translational Repression in Adult Transgenic Mice. Mol. Cell. Biol. *21*, 1833–1840.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121–134.

Dan, H.C., Adli, M., and Baldwin, A.S. (2007). Regulation of mammalian target of rapamycin activity in PTEN-inactive prostate cancer cells by I kappa B kinase alpha. Cancer Res. *67*, 6263–6269.

Datta, B., Datta, R., Mukherjee, S., and Zhang, Z. (1999). Increased phosphorylation of eukaryotic initiation factor 2alpha at the G2/M boundary in human osteosarcoma cells correlates with deglycosylation of p67 and a decreased rate of protein synthesis. Exp. Cell Res. *250*, 223–230.

Dazert, E., and Hall, M.N. (2011). MTOR signaling in disease. Curr. Opin. Cell Biol. *23*, 744–755.

De Breyne, S., Yu, Y., Unbehaun, A., Pestova, T.V., and Hellen, C.U.T. (2009). Direct functional interaction of initiation factor eIF4G with type 1 internal ribosomal entry sites. Proc. Natl. Acad. Sci. *106*, 9197–9202.

De Sousa Abreu, R., Penalva, L.O., Marcotte, E.M., and Vogel, C. (2009). Global signatures of protein and mRNA expression levels. Mol. Biosyst. *5*, 1512–1526.

Dever, T.E. (2002). Gene-specific regulation by general translation factors. Cell *108*, 545–556.

Dever, T.E., and Hinnebusch, A.G. (2005). GCN2 whets the appetite for amino acids. Mol. Cell *18*, 141–142.

Deldicque, L., Bertrand, L., Patton, A., Francaux, M., and Baar, K. (2011). ER stress induces anabolic resistance in muscle cells through PKB-induced blockade of MTORC1. PloS One *6*, e20993.

DeLeo, A.B., Jay, G., Appella, E., Dubois, G.C., Law, L.W., and Old, L.J. (1979). Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc. Natl. Acad. Sci. U. S. A. *76*, 2420–2424.

Dennis, M.D., Jefferson, L.S., and Kimball, S.R. (2012). Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 proteins in the regulation of protein synthesis. J. Biol. Chem. *287*, 42890–42899.

Demontis, F., and Perrimon, N. (2010). FOXO/4E-BP Signaling in Drosophila Muscles Regulates Organism-wide Proteostasis during Aging. Cell *143*, 813–825.

DeYoung, M.P., Horak, P., Sofer, A., Sgroi, D., and Ellisen, L.W. (2008). Hypoxia regulates TSC1/2-MTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev. *22*, 239–251.

Di Nardo, A., Kramvis, I., Cho, N., Sadowski, A., Meikle, L., Kwiatkowski, D.J., and Sahin, M. (2009). Tuberous sclerosis complex activity is required to control neuronal stress responses in an MTOR-dependent manner. J. Neurosci. Off. J. Soc. Neurosci. *29*, 5926–5937.

Dibble, C.C., Asara, J.M., and Manning, B.D. (2009). Characterization of Rictor phosphorylation sites reveals direct regulation of MTOR complex 2 by S6K1. Mol. Cell. Biol. *29*, 5657–5670.

Dibble, C.C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J.M., Finan, P.M., Kwiatkowski, D.J., Murphy, L.O., and Manning, B.D. (2012). TBC1D7 Is a Third Subunit of the TSC1-TSC2 Complex Upstream of MTORC1. Mol. Cell *47*, 535–546.

Dmitriev, S.E., Terenin, I.M., Dunaevsky, Y.E., Merrick, W.C., and Shatsky, I.N. (2003). Assembly of 48S Translation Initiation Complexes from Purified Components with mRNAs That Have Some Base Pairing within Their 5′ Untranslated Regions. Mol. Cell. Biol. *23*, 8925–8933.

Dmitriev, S.E., Terenin, I.M., Andreev, D.E., Ivanov, P.A., Dunaevsky, J.E., Merrick, W.C., and Shatsky, I.N. (2010). GTP-independent tRNA delivery to the ribosomal P-site by a novel eukaryotic translation factor. J Biol Chem *285*, 26779–26787.

Dobbyn, H.C., Hill, K., Hamilton, T.L., Spriggs, K.A., Pickering, B.M., Coldwell, M.J., de Moor, C.H., Bushell, M., and Willis, A.E. (2008). Regulation of BAG1 IRES-mediated translation following chemotoxic stress. Oncogene *27*, 1167–1174.

Dobrikova, E., Florez, P., Bradrick, S., and Gromeier, M. (2003). Activity of a type 1 picornavirus internal ribosomal entry site is determined by sequences within the 3′ nontranslated region. Proc. Natl. Acad. Sci. *100*, 15125–15130.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr, Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Donzé, O., Jagus, R., Koromilas, A.E., Hershey, J.W., and Sonenberg, N. (1995). Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells. EMBO J. *14*, 3828–3834.

Dorokhov, Y.L., Skulachev, M.V., Ivanov, P.A., Zvereva, S.D., Tjulkina, L.G., Merits, A., Gleba, Y.Y., Hohn, T., and Atabekov, J.G. (2002). Polypurine (A)-rich sequences promote cross-kingdom conservation of internal ribosome entry. Proc. Natl. Acad. Sci. U. S. A. *99*, 5301–5306.

Dorrello, N.V., Peschiaroli, A., Guardavaccaro, D., Colburn, N.H., Sherman, N.E., and Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science *314*, 467–471.

Dostie, J., Ferraiuolo, M., Pause, A., Adam, S.A., and Sonenberg, N. (2000). A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5[prime] cap-binding protein, eIF4E. EMBO J *19*, 3142–3156.

Dotsch, V., Bernassola, F., Coutandin, D., Candi, E., and Melino, G. (2010). p63 and p73, the Ancestors of p53. Cold Spring Harb. Perspect. Biol. *2*, a004887–a004887.

Dowling, R.J.O., Topisirovic, I., Alain, T., Bidinosti, M., Fonseca, B.D., Petroulakis, E., Wang, X., Larsson, O., Selvaraj, A., Liu, Y., et al. (2010). MTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science *328*, 1172–1176.

Du, K., Herzig, S., Kulkarni, R.N., and Montminy, M. (2003). TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science *300*, 1574–1577.

Duncan, R., Milburn, S.C., and Hershey, J.W. (1987). Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. J. Biol. Chem. *262*, 380–388.

el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. Nat. Genet. *1*, 45–49.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Easton, J.B., and Houghton, P.J. (2006). MTOR and cancer therapy. Oncogene *25*, 6436–6446.

Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Skurnik, Y., Arai, N., Rotter, V., and Oren, M. (1988). Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene *3*, 313–321.

Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O., and Oren, M. (1989). Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. U. S. A. *86*, 8763–8767.

Evdokimova, V., Tognon, C., Ng, T., Ruzanov, P., Melnyk, N., Fink, D., Sorokin, A., Ovchinnikov, L.P., Davicioni, E., Triche, T.J., et al. (2009). Translational activation of snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition. Cancer Cell *15*, 402–415.

Everson, W.V., Flaim, K.E., Susco, D.M., Kimball, S.R., and Jefferson, L.S. (1989). Effect of amino acid deprivation on initiation of protein synthesis in rat hepatocytes. Am. J. Physiol. - Cell Physiol. *256*, C18–C27.

Facchinetti, V., Ouyang, W., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A.C., Mao, Y., Miao, R.Q., et al. (2008). The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J. *27*, 1932–1943.

Finlay, C.A., Hinds, P.W., Tan, T.H., Eliyahu, D., Oren, M., and Levine, A.J. (1988). Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol. Cell. Biol. *8*, 531–539.

Faivre, S., Kroemer, G., and Raymond, E. (2006). Current development of MTOR inhibitors as anticancer agents. Nat. Rev. Drug Discov. *5*, 671–688.

Fearnhead, N.S., Britton, M.P., and Bodmer, W.F. (2001). The ABC of APC. Hum. Mol. Genet. *10*, 721–733.

Feldman, M.E., Apsel, B., Uotila, A., Loewith, R., Knight, Z.A., Ruggero, D., and Shokat, K.M. (2009). Active-Site Inhibitors of MTOR Target Rapamycin-Resistant Outputs of MTORC1 and MTORC2. PLoS Biol. *7*, e38.

Feng, Z., Zhang, H., Levine, A.J., and Jin, S. (2005). The coordinate regulation of the p53 and MTOR pathways in cells. Proc. Natl. Acad. Sci. U. S. A. *102*, 8204–8209.

Feng, Z., Hu, W., de Stanchina, E., Teresky, A.K., Jin, S., Lowe, S., and Levine, A.J. (2007). The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-MTOR pathways. Cancer Res. *67*, 3043–3053.

Fernandez, J., Yaman, I., Sarnow, P., Snider, M.D., and Hatzoglou, M. (2002). Regulation of internal ribosomal entry site-mediated translation by phosphorylation of the translation initiation factor eIF2alpha. J. Biol. Chem. *277*, 19198–19205.

Fernandez, J., Yaman, I., Huang, C., Liu, H., Lopez, A.B., Komar, A.A., Caprara, M.G., Merrick, W.C., Snider, M.D., Kaufman, R.J., et al. (2005). Ribosome stalling regulates IRES-mediated translation in eukaryotes, a parallel to prokaryotic attenuation. Mol. Cell *17*, 405–416.

Ferraiuolo, M.A., Basak, S., Dostie, J., Murray, E.L., Schoenberg, D.R., and Sonenberg, N. (2005). A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. J. Cell Biol. *170*, 913–924.

Fontoura, B.M., Atienza, C.A., Sorokina, E.A., Morimoto, T., and Carroll, R.B. (1997). Cytoplasmic p53 polypeptide is associated with ribosomes. Mol. Cell. Biol. *17*, 3146–3154.

Foukas, L.C., and Shepherd, P.R. (2004). eIF4E binding protein 1 and H-Ras are novel substrates for the protein kinase activity of class-I phosphoinositide 3-kinase. Biochemical and Biophysical Research Communications *319*, 541–549.

Fox, J.T., and Stover, P.J. (2009). Mechanism of the Internal Ribosome Entry Site-mediated Translation of Serine Hydroxymethyltransferase 1. J. Biol. Chem. *284*, 31085–31096.

Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A., and Sabatini, D.M. (2006). mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct MTORC2s. Curr. Biol. CB *16*, 1865–1870.

Fujita, K., Mondal, A.M., Horikawa, I., Nguyen, G.H., Kumamoto, K., Sohn, J.J., Bowman, E.D., Mathe, E.A., Schetter, A.J., Pine, S.R., et al. (2009). p53 isoforms Δ133p53 and p53β are endogenous regulators of replicative cellular senescence. Nat. Cell Biol. *11*, 1135–1142.

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992). A transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol. *12*, 2866–2871.

Gaccioli, F., Huang, C.C., Wang, C., Bevilacqua, E., Franchi-Gazzola, R., Gazzola, G.C., Bussolati, O., Snider, M.D., and Hatzoglou, M. (2006). Amino acid starvation induces the SNAT2 neutral amino acid transporter by a mechanism that involves eukaryotic initiation factor 2alpha phosphorylation and cap-independent translation. J. Biol. Chem. *281*, 17929–17940.

Gadéa, G., Lapasset, L., Gauthier-Rouvière, C., and Roux, P. (2002). Regulation of Cdc42-mediated morphological effects: a novel function for p53. EMBO J. *21*, 2373–2382.

Gajjar, M., Candeias, M.M., Malbert-Colas, L., Mazars, A., Fujita, J., Olivares-Illana, V., and Fåhraeus, R. (2012). The p53 mRNA-Mdm2 Interaction Controls Mdm2 Nuclear Trafficking and Is Required for p53 Activation following DNA Damage. Cancer Cell *21*, 25–35.

Garami, A., Zwartkruis, F.J.T., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S.C., Hafen, E., Bos, J.L., and Thomas, G. (2003). Insulin activation of Rheb, a mediator of MTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol. Cell *11*, 1457–1466.

García-Martínez, J.M., and Alessi, D.R. (2008). MTOR complex 2 (MTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem. J. *416*, 375–385.

Gardner, L., Lee, L., and Dang, C. (2002). The c-Myc oncogenic transcription factor. Encyclopedia of Cancer *2*.

Gatz, S.A., and Wiesmüller, L. (2006). p53 in recombination and repair. Cell Death Differ. *13*, 1003–1016.

Ge, J., and Yu, Y.-T. (2013). RNA pseudouridylation: new insights into an old modification. Trends Biochem. Sci. *38*, 210–218.

Gerlitz, G., Jagus, R., and Elroy-Stein, O. (2002). Phosphorylation of initiation factor-2α is required for activation of internal translation initiation during cell differentiation. Eur. J. Biochem. *269*, 2810–2819.

Ghosh, A., Stewart, D., and Matlashewski, G. (2004). Regulation of Human p53 Activity and Cell Localization by Alternative Splicing. Mol. Cell. Biol. *24*, 7987–7997.

Gilbert, W.V., Zhou, K., Butler, T.K., and Doudna, J.A. (2007). Cap-Independent Translation Is Required for Starvation-Induced Differentiation in Yeast. Science *317*, 1224–1227.

Goss, K.H., Trzepacz, C., Tuohy, T.M., and Groden, J. (2002). Attenuated APC alleles produce functional protein from internal translation initiation. Proc. Natl. Acad. Sci. *99*, 8161–8166.

Graber, T.E., and Holcik, M. (2007). Cap-independent regulation of gene expression in apoptosis. Mol. Biosyst. *3*, 825.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. Cell *66*, 589–600.

Gross, J.D., Moerke, N.J., von der Haar, T., Lugovskoy, A.A., Sachs, A.B., McCarthy, J.E.G., and Wagner, G. (2003). Ribosome Loading onto the mRNA Cap Is Driven by Conformational Coupling between eIF4G and eIF4E. Cell *115*, 739–750.

Grover, R., Sharathchandra, A., Ponnuswamy, A., Khan, D., and Das, S. (2011). Effect of mutations on the p53 IRES RNA structure: implications for de-regulation of the synthesis of p53 isoforms. RNA Biol *8*, 132–142.

Grover, R., Ray, P.S., and Das, S. (2008). Polypyrimidine tract binding protein regulates IRES-mediated translation of p53 isoforms. Cell Cycle *7*, 2189–2198.

Grover, R., Candeias, M.M., Faahraeus, R., and Das, S. (2009). p53 and little brother p53/47: linking IRES activities with protein functions. Oncogene *28*, 2766–2772.

Gu, B., and Zhu, W.-G. (2012). Surf the post-translational modification network of p53 regulation. Int. J. Biol. Sci. *8*, 672–684.

Guo, B., Phillips, J.D., Yu, Y., and Leibold, E.A. (1995). Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. J. Biol. Chem. *270*, 21645–21651.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008). AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. Mol. Cell *30*, 214–226.

Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J. *14*, 5701.

Haghighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T., and Sonenberg, N. (1996). The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. J. Virol. *70*, 8444–8450.

Hara, K., Yonezawa, K., Kozlowski, M.T., Sugimoto, T., Andrabi, K., Weng, Q.P., Kasuga, M., Nishimoto, I., and Avruch, J. (1997). Regulation of eIF-4E BP1 phosphorylation by MTOR. J. Biol. Chem. *272*, 26457–26463.

Hara, K., Yonezawa, K., Weng, Q.P., Kozlowski, M.T., Belham, C., and Avruch, J. (1998). Amino acid sufficiency and MTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J. Biol. Chem. *273*, 14484–14494.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., and Yonezawa, K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell *110*, 177–189.

Harding, H.P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature *397*, 271–274.

Harding, H.P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000a). Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol. Cell *5*, 897–904.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000b). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell *6*, 1099–1108.

Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., and Paules, R. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell *11*, 619–633.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., and Swindell, E. (1995). Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell *6*, 387–400.

Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R., et al. (2004). The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J. Cell Biol. *166*, 213–223.

Harris, T.E., Chi, A., Shabanowitz, J., Hunt, D.F., Rhoads, R.E., and Lawrence, J.C. (2006). MTOR-dependent stimulation of the association of eIF4G and eIF3 by insulin. EMBO J. *25*, 1659–1668.

Hauge, C., Antal, T.L., Hirschberg, D., Doehn, U., Thorup, K., Idrissova, L., Hansen, K., Jensen, O.N., Jørgensen, T.J., Biondi, R.M., et al. (2007). Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation. EMBO J. *26*, 2251–2261.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of MTOR. Genes Dev. *18*, 1926.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and

activated by proteolysis in response to endoplasmic reticulum stress. Mol. Biol. Cell *10*, 3787–3799.

Haze, K., Okada, T., Yoshida, H., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2001). Identification of the G13 (cAMP-response-element-binding protein-related protein) gene product related to activating transcription factor 6 as a transcriptional activator of the mammalian unfolded protein response. Biochem. J. *355*, 19–28.

Heifetz, A., Keenan, R.W., and Elbein, A.D. (1979). Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl-phosphate Glc-NAc-1-phosphate transferase. Biochemistry (Mosc.) *18*, 2186–2192.

Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*, 905–909.

Hellen, C.U., and Sarnow, P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev. *15*, 1593–1612.

Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W., and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol. Cell *1*, 3–11.

Hernández, G., Vázquez-Pianzola, P., Sierra, J.M., and Rivera-Pomar, R. (2004). Internal ribosome entry site drives cap-independent translation of reaper and heat shock protein 70 mRNAs in Drosophila embryos. RNA *10*, 1783–1797.

Hernández-Negrete, I., Carretero-Ortega, J., Rosenfeldt, H., Hernández-García, R., Calderón-Salinas, J.V., Reyes-Cruz, G., Gutkind, J.S., and Vázquez-Prado, J. (2007). P-Rex1 links mammalian target of rapamycin signaling to Rac activation and cell migration. J. Biol. Chem. *282*, 23708–23715.

Hernegger, G.S., Moore, H.G., and Guillem, J.G. (2002). Attenuated familial adenomatous polyposis: an evolving and poorly understood entity. Dis. Colon Rectum *45*, 127–134; discussion 134–136.

Hinnebusch, A.G. (2006). eIF3: a versatile scaffold for translation initiation complexes. Trends Biochem. Sci. *31*, 553–562.

Hinton, T.M., Coldwell, M.J., Carpenter, G.A., Morley, S.J., and Pain, V.M. (2007). Functional Analysis of Individual Binding Activities of the Scaffold Protein eIF4G. J. Biol. Chem. *282*, 1695–1708.

Hoffman, B., and Liebermann, D.A. (1998). The proto-oncogene c-myc and apoptosis. Oncogene *17*, 3351–3357.

Hofstetter, G., Berger, A., Berger, R., Zorić, A., Braicu, E.I., Reimer, D., Fiegl, H., Marth, C., Zeimet, A.G., Ulmer, H., et al. (2012). The N-terminally truncated p53 isoform Δ40p53 influences prognosis in mucinous ovarian cancer. Int. J. Gynecol. Cancer Off. J. Int. Gynecol. Cancer Soc. *22*, 372–379.

Hofstetter, G., Berger, A., Fiegl, H., Slade, N., Zorić, A., Holzer, B., Schuster, E., Mobus, V.J., Reimer, D., Daxenbichler, G., et al. (2010). Alternative splicing of p53 and p73: the novel p53 splice variant p53delta is an independent prognostic marker in ovarian cancer. Oncogene *29*, 1997–2004.

Holcik, M., Lefebvre, C., Yeh, C., Chow, T., and Korneluk, R.G. (1999). A new internal-ribosome-entry-site motif potentiates XIAP-mediated cytoprotection. Nat. Cell Biol. *1*, 190–192.

Holcik, M., Sonenberg, N., and Korneluk, R.G. (2000a). Internal ribosome initiation of translation and the control of cell death. Trends Genet. *16*, 469–473.

Holcik, M., Yeh, C., Korneluk, R.G., and Chow, T. (2000b). Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene *19*, 4174–4177.

Hollien, J., Lin, J.H., Li, H., Stevens, N., Walter, P., and Weissman, J.S. (2009). Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J. Cell Biol. *186*, 323–331.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). MTOR and S6K1 Mediate Assembly of the Translation Preinitiation Complex through Dynamic Protein Interchange and Ordered Phosphorylation Events. Cell *123*, 569–580.

Honda, M., Ping, L.H., Rijnbrand, R.C., Amphlett, E., Clarke, B., Rowlands, D., and Lemon, S.M. (1996). Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. Virology *222*, 31–42.

Hope, N.R., and Murray, G.I. (2010). The expression profile of RNA-binding proteins in primary and metastatic colorectal cancer: relationship of heterogeneous nuclear ribonucleoproteins with prognosis. Hum. Pathol.

Hornstein, E., Tang, H., and Meyuhas, O. (2001). Mitogenic and nutritional signals are transduced into translational efficiency of TOP mRNAs. Cold Spring Harb. Symp. Quant. Biol. *66*, 477–484.

Hresko, R.C., and Mueckler, M. (2005). MTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J. Biol. Chem. *280*, 40406–40416.

Hsieh, A.C., Liu, Y., Edlind, M.P., Ingolia, N.T., Janes, M.R., Sher, A., Shi, E.Y., Stumpf, C.R., Christensen, C., Bonham, M.J., et al. (2012). The translational landscape of MTOR signalling steers cancer initiation and metastasis. Nature *485*, 55–61.

Hsu, P.P., Kang, S.A., Rameseder, J., Zhang, Y., Ottina, K.A., Lim, D., Peterson, T.R., Choi, Y., Gray, N.S., Yaffe, M.B., et al. (2011). The MTOR-regulated phosphoproteome reveals a mechanism of MTORC1-mediated inhibition of growth factor signaling. Science *332*, 1317–1322.

Huang, J., and Manning, B.D. (2009). A complex interplay between Akt, TSC2 and the two MTOR complexes. Biochem. Soc. Trans. *37*, 217–222.

Huang, J., Dibble, C.C., Matsuzaki, M., and Manning, B.D. (2008). The TSC1-TSC2 complex is required for proper activation of MTOR complex 2. Mol. Cell. Biol. *28*, 4104–4115.

Huang, J., Wu, S., Wu, C.-L., and Manning, B.D. (2009). Signaling events downstream of mammalian target of rapamycin complex 2 are attenuated in cells and tumors deficient for the tuberous sclerosis complex tumor suppressors. Cancer Res. *69*, 6107–6114.

Hudson, C.C., Liu, M., Chiang, G.G., Otterness, D.M., Loomis, D.C., Kaper, F., Giaccia, A.J., and Abraham, R.T. (2002). Regulation of Hypoxia-Inducible Factor 1 Expression and Function by the Mammalian Target of Rapamycin. Mol. Cell. Biol. *22*, 7004–7014.

Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K.-L. (2008). Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J. *27*, 1919–1931.

Imataka, H., Olsen, H.S., and Sonenberg, N. (1997). A new translational regulator with homology to eukaryotic translation initiation factor 4G. EMBO J. *16*, 817–825.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.-L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses MTOR signalling. Nat. Cell Biol. *4*, 648–657.

Inoki, K., Li, Y., Xu, T., and Guan, K.-L. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulates MTOR signaling. Genes Dev. *17*, 1829–1834.

Inoki, K., Ouyang, H., Zhu, T., Lindvall, C., Wang, Y., Zhang, X., Yang, Q., Bennett, C., Harada, Y., Stankunas, K., et al. (2006). TSC2 integrates Wnt and energy signals

via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell *126*, 955–968.

Ito, E., Yanagisawa, Y., Iwahashi, Y., Suzuki, Y., Nagasaki, H., Akiyama, Y., Sugano, S., Yuasa, Y., and Maruyama, K. (1999). A Core Promoter and a Frequent Single-Nucleotide Polymorphism of the Mismatch Repair GenehMLH1. Biochem. Biophys. Res. Commun. *256*, 488–494.

Jaag, H.M., Kawchuk, L., Rohde, W., Fischer, R., Emans, N., and Prüfer, D. (2003). An unusual internal ribosomal entry site of inverted symmetry directs expression of a potato leafroll polerovirus replication-associated protein. Proc. Natl. Acad. Sci. *100*, 8939–8944.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol. *6*, 1122–1128.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-MTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell *127*, 125–137.

Jack, K., Bellodi, C., Landry, D.M., Niederer, R.O., Meskauskas, A., Musalgaonkar, S., Kopmar, N., Krasnykh, O., Dean, A.M., Thompson, S.R., et al. (2011). rRNA Pseudouridylation Defects Affect Ribosomal Ligand Binding and Translational Fidelity from Yeast to Human Cells. Molecular Cell *44*, 660–666.

Jackson, R.J., Hellen, C.U.T., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nat. Rev. Mol. Cell Biol. *11*, 113–127.

Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature *461*, 1071–1078.

Jacobson, B.A., Alter, M.D., Kratzke, M.G., Frizelle, S.P., Zhang, Y., Peterson, M.S., Avdulov, S., Mohorn, R.P., Whitson, B.A., Bitterman, P.B., et al. (2006). Repression of Cap-Dependent Translation Attenuates the Transformed Phenotype in Non–Small Cell Lung Cancer Both In vitro and In vivo. Cancer Res. *66*, 4256.

Janes, M.R., Vu, C., Mallya, S., Shieh, M.P., Limon, J.J., Li, L.-S., Jessen, K.A., Martin, M.B., Ren, P., Lilly, M.B., et al. (2013). Efficacy of the investigational MTOR kinase inhibitor MLN0128/INK128 in models of B-cell acute lymphoblastic leukemia. Leukemia *27*, 586–594.

Jang, C.J., and Jan, E. (2010). Modular domains of the Dicistroviridae intergenic internal ribosome entry site. RNA New York N *16*, 1182–1195.

Jang, S.K., and Wimmer, E. (1990). Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. *4*, 1560–1572.

Jang, S.K., Kräusslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C., and Wimmer, E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. *62*, 2636–2643.

Jefferies, H.B., Reinhard, C., Kozma, S.C., and Thomas, G. (1994). Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc. Natl. Acad. Sci. U. S. A. *91*, 4441–4445.

Jen, J., Powell, S.M., Papadopoulos, N., Smith, K.J., Hamilton, S.R., Vogelstein, B., and Kinzler, K.W. (1994). Molecular determinants of dysplasia in colorectal lesions. Cancer Res. *54*, 5523–5526.

Jiang, X., and Wang, X. (2000). Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to APAF1. J. Biol. Chem. *275*, 31199–31203.

Johannes, G., and Sarnow, P. (1998). Cap-independent polysomal association of natural mRNAs encoding c-myc, BiP, and eIF4G conferred by internal ribosome entry sites. RNA New York N *4*, 1500–1513.

Johannes, G., Carter, M.S., Eisen, M.B., Brown, P.O., and Sarnow, P. (1999). Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. Proc. Natl. Acad. Sci. U. S. A. *96*, 13118.

Johnson, T.M., Yu, Z.X., Ferrans, V.J., Lowenstein, R.A., and Finkel, T. (1996). Reactive oxygen species are downstream mediators of p53-dependent apoptosis. Proc. Natl. Acad. Sci. U. S. A. *93*, 11848–11852.

Jopling, C.L., Spriggs, K.A., Mitchell, S.A., Stoneley, M., and Willis, A.E. (2004). L-Myc protein synthesis is initiated by internal ribosome entry. RNA *10*, 287–298.

Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., et al. (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. Cell *66*, 601–613.

Kahn, B.B., Alquier, T., Carling, D., and Hardie, D.G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. *1*, 15–25.

Kaizuka, T., Hara, T., Oshiro, N., Kikkawa, U., Yonezawa, K., Takehana, K., Iemura, S.-I., Natsume, T., and Mizushima, N. (2010). Tti1 and Tel2 are critical factors in mammalian target of rapamycin complex assembly. J. Biol. Chem. *285*, 20109–20116.

Kaminski, A., Howell, M.T., and Jackson, R.J. (1990). Initiation of encephalomyocarditis virus RNA translation: the authentic initiation site is not selected by a scanning mechanism. EMBO J. *9*, 3753–3759.

Kaminski, A., Belsham, G., and Jackson, R.J. (1994). Translation of encephalomyocarditis virus RNA: parameters influencing the selection of the internal initiation site. EMBO J. *13*, 1673.

Kang, Y.J., Lu, M.K., and Guan, K.L. (2010). The TSC1 and TSC2 tumor suppressors are required for proper ER stress response and protect cells from ER stress-induced apoptosis. Cell Death Differ. *18*, 133–144.

Kannan, K., Kaminski, N., Rechavi, G., Jakob-Hirsch, J., Amariglio, N., and Givol, D. (2001). DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of APAF1. Oncogene *20*, 3449–3455.

Kantidakis, T., Ramsbottom, B.A., Birch, J.L., Dowding, S.N., and White, R.J. (2010). MTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. Proc. Natl. Acad. Sci. U. S. A. *107*, 11823–11828.

Kapasi, P., Chaudhuri, S., Vyas, K., Baus, D., Komar, A.A., Fox, P.L., Merrick, W.C., and Mazumder, B. (2007). L13a Blocks 48S Assembly: Role of a General Initiation Factor in mRNA-Specific Translational Control. Mol. Cell *25*, 113–126.

Kato, H., Nakajima, S., Saito, Y., Takahashi, S., Katoh, R., and Kitamura, M. (2011). MTORC1 serves ER stress-triggered apoptosis via selective activation of the IRE1–JNK pathway. Cell Death Differ. *19*, 310–320.

Kato, H., Katoh, R., and Kitamura, M. (2013). Dual Regulation of Cadmium-Induced Apoptosis by MTORC1 through Selective Induction of IRE1 Branches in Unfolded Protein Response. PLoS ONE *8*, e64344.

Kawauchi, K., Araki, K., Tobiume, K., and Tanaka, N. (2008). p53 regulates glucose metabolism through an IKK-NF-kappaB pathway and inhibits cell transformation. Nat. Cell Biol. *10*, 611–618.

Kaye, N.M., Emmett, K.J., Merrick, W.C., and Jankowsky, E. (2009). Intrinsic RNA Binding by the Eukaryotic Initiation Factor 4F Depends on a Minimal RNA Length but Not on the m7G Cap. J. Biol. Chem. *284*, 17742–17750.

Keller, R.K., Boon, D.Y., and Crum, F.C. (1979). N-Acetylglucosamine- 1 -phosphate transferase from hen oviduct: solubilization, characterization, and inhibition by tunicamycin. Biochemistry (Mosc.) *18*, 3946–3952.

Kemp, B.E., Mitchelhill, K.I., Stapleton, D., Michell, B.J., Chen, Z.-P., and Witters, L.A. (1999). Dealing with energy demand: the AMP-activated protein kinase. Trends Biochem. Sci. *24*, 22–25.

Khan, D., Sharathchandra, A., Ponnuswamy, A., Grover, R., and Das, S. (2012). Effect of a natural mutation in the 5[prime] untranslated region on the translational control of p53 mRNA. Oncogene.

Khoury, M.P., and Bourdon, J.-C. (2009). The Isoforms of the p53 Protein. Cold Spring Harb. Perspect. Biol. *2*, a000927–a000927.

Kim, D.-H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). MTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163–175.

Kim, D.-H., Sarbassov, D.D., Ali, S.M., Latek, R.R., Guntur, K.V.P., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2003). GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and MTOR. Mol. Cell *11*, 895–904.

Kim, D.-Y., Kim, W., Lee, K.-H., Kim, S.-H., Lee, H.-R., Kim, H.-J., Jung, Y., Choi, J.-H., and Kim, K.-T. (2013). hnRNP Q regulates translation of p53 in normal and stress conditions. Cell Death Differ *20*, 226–234.

Kim, J.H., Park, S.M., Park, J.H., Keum, S.J., and Jang, S.K. (2011). eIF2A mediates translation of hepatitis C viral mRNA under stress conditions. EMBO J *30*, 2454–2464.

Kim, S.J., DeStefano, M.A., Oh, W.J., Wu, C., Vega-Cotto, N.M., Finlan, M., Liu, D., Su, B., and Jacinto, E. (2012). MTOR Complex 2 Regulates Proper Turnover of Insulin Receptor Substrate-1 via the Ubiquitin Ligase Subunit Fbw8. Molecular Cell *48*, 875–887.

Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K., Hara, K., Kemp, B.E., Witters, L.A., Mimura, O., and Yonezawa, K. (2003). A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (MTOR) signalling pathway. Genes Cells Devoted Mol. Cell. Mech. *8*, 65–79.

Kinzler, K., Nilbert, M., Su, L., Vogelstein, B., Bryan, T., Levy, D., Smith, K., Preisinger, A., Hedge, P., McKechnie, D., et al. (1991). Identification of FAP locus genes from chromosome 5q21. Science *253*, 661–665.

Kochetov, A.V., Palyanov, A., Titov, I.I., Grigorovich, D., Sarai, A., and Kolchanov, N.A. (2007). AUG_hairpin: prediction of a downstream secondary structure influencing the recognition of a translation start site. BMC Bioinformatics *8*, 318.

Kolupaeva, V.G., Unbehaun, A., Lomakin, I.B., Hellen, C.U.T., and Pestova, T.V. (2005). Binding of eukaryotic initiation factor 3 to ribosomal 40S subunits and its role in ribosomal dissociation and anti-association. RNA *11*, 470–486.

Komar, A.A., and Hatzoglou, M. (2005). Internal ribosome entry sites in cellular mRNAs: mystery of their existence. J. Biol. Chem. *280*, 23425–23428.

Komar, A.A., and Hatzoglou, M. (2011). Cellular IRES-mediated translation: The war of ITAFs in pathophysiological states. Cc *10*, 229–240.

Koritzinsky, M., Magagnin, M.G., van den Beucken, T., Seigneuric, R., Savelkouls, K., Dostie, J., Pyronnet, S., Kaufman, R.J., Weppler, S.A., Voncken, J.W., et al. (2006). Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control. EMBO J. *25*, 1114–1125.

Koritzinsky, M., Rouschop, K., Vandenbeucken, T., Magagnin, M., Savelkouls, K., Lambin, P., and Wouters, B. (2007). Phosphorylation of eIF2α is required for mRNA translation inhibition and survival during moderate hypoxia. Radiother. Oncol. *83*, 353–361.

Koromilas, A.E., Lazaris-Karatzas, A., and Sonenberg, N. (1992a). mRNAs containing extensive secondary structure in their 5'non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. The EMBO Journal *11*, 4153.

Koromilas, A.E., Roy, S., Barber, G.N., Katze, M.G., and Sonenberg, N. (1992b). Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. Science *257*, 1685–1689.

Korotchkina, L.G., Leontieva, O.V., Bukreeva, E.I., Demidenko, Z.N., Gudkov, A.V., and Blagosklonny, M.V. (2010). The choice between p53-induced senescence and quiescence is determined in part by the MTOR pathway. Aging (Albany NY) *2*, 344–352.

Kortlever, R.M., Higgins, P.J., and Bernards, R. (2006). Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. Nat. Cell Biol. *8*, 877–884.

Koumenis, C., Naczki, C., Koritzinsky, M., Rastani, S., Diehl, A., Sonenberg, N., Koromilas, A., and Wouters, B.G. (2002). Regulation of Protein Synthesis by Hypoxia via Activation of the Endoplasmic Reticulum Kinase PERK and Phosphorylation of the Translation Initiation Factor eIF2. Mol. Cell. Biol. *22*, 7405–7416.

Kozak, M. (1978). How do eucaryotic ribosomes select initiation regions in messenger RNA? Cell *15*, 1109–1123.

Kozak, M. (1984). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. Nature *308*, 241–246.

Kozak, M. (1986a). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell *44*, 283–292.

Kozak, M. (1986b). Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. U.S.A. *83*, 2850–2854.

Kozak, M. (1987a). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. *15*, 8125–8148.

Kozak, M. (1987b). Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. Mol. Cell. Biol. *7*, 3438–3445.

Kozak, M. (1989a). Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. Mol. Cell. Biol. *9*, 5073–5080.

Kozak, M. (1989b). Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. Mol. Cell. Biol. *9*, 5134–5142.

Kozak, M. (1990). Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. Proc. Natl. Acad. Sci. U.S.A. *87*, 8301–8305.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem *266*, 19867–19870.

Kozak, M. (2001). Constraints on reinitiation of translation in mammals. Nucleic Acids Res. *29*, 5226–5232.

Knudsen, A.L., Bisgaard, M.L., and Bülow, S. (2003). Attenuated familial adenomatous polyposis (AFAP). A review of the literature. Fam. Cancer *2*, 43–55.

Kress, M., May, E., Cassingena, R., and May, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J. Virol. *31*, 472–483.

Kunz, C., Pebler, S., Otte, J., and von der Ahe, D. (1995). Differential regulation of plasminogen activator and inhibitor gene transcription by the tumor suppressor p53. Nucleic Acids Res. *23*, 3710–3717.

Land, S.C., and Tee, A.R. (2007). Hypoxia-inducible Factor 1 Is Regulated by the Mammalian Target of Rapamycin (MTOR) via an MTOR Signaling Motif. J. Biol. Chem. *282*, 20534–20543.

Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature *278*, 261–263.

Lang, K.J.., Kappel, A., and Goodall, G.J. (2002). Hypoxia-inducible factor-1alpha mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. Mol. Biol. Cell *13*, 1792.

Laplante, M., and Sabatini, D.M. (2012). MTOR Signaling in Growth Control and Disease. Cell *149*, 274–293.

Laplante, M., and Sabatini, D.M. (2013). Regulation of MTORC1 and its impact on gene expression at a glance. J. Cell Sci. *126*, 1713–1719.

Laronga, C., Yang, H.Y., Neal, C., and Lee, M.H. (2000). Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. J. Biol. Chem. *275*, 23106–23112.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P.C., and Semenza, G.L. (2001). HER2 (neu) Signaling Increases the Rate of Hypoxia-Inducible Factor 1 (HIF-1) Synthesis: Novel Mechanism for HIF-1-Mediated Vascular Endothelial Growth Factor Expression. Mol. Cell. Biol. *21*, 3995–4004.

Le Hir, H., Moore, M.J., and Maquat, L.E. (2000). Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon-exon junctions. Genes Dev. *14*, 1098–1108.

Le Quesne, J.P.., Stoneley, M., Fraser, G.A., and Willis, A.E. (2001). Derivation of a structural model for the c-myc IRES1. Journal of Molecular Biology *310*, 111–126.

Le, S.-Y., and Maizel, J.V. (1997). A common RNA structural motif involved in the internal initiation of translation of cellular mRNAs. Nucleic Acids Res. *25*, 362–369.

Leal, J.F.M., Fominaya, J., Cascón, A., Guijarro, M.V., Blanco-Aparicio, C., Lleonart, M., Castro, M.E., Ramon Y Cajal, S., Robledo, M., Beach, D.H., et al. (2008). Cellular senescence bypass screen identifies new putative tumor suppressor genes. Oncogene *27*, 1961–1970.

Lee, D.H., and Goldberg, A.L. (1998). Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. *8*, 397–403.

Lee, F., and Yanofsky, C. (1977). Transcription termination at the trp operon attenuators of Escherichia coli and Salmonella typhimurium: RNA secondary structure and regulation of termination. Proc. Natl. Acad. Sci. U. S. A. *74*, 4365–4369.

Lee, A.-H., Iwakoshi, N.N., and Glimcher, L.H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol. Cell. Biol. *23*, 7448–7459.

Lee, D.-F., Kuo, H.-P., Chen, C.-T., Hsu, J.-M., Chou, C.-K., Wei, Y., Sun, H.-L., Li, L.-Y., Ping, B., Huang, W.-C., et al. (2007). IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the MTOR pathway. Cell *130*, 440–455.

LeFebvre, A.K., Korneeva, N.L., Trutschl, M., Cvek, U., Duzan, R.D., Bradley, C.A., Hershey, J.W., and Rhoads, R.E. (2006). Translation initiation factor eIF4G-1 binds to eIF3 through the eIF3e subunit. J. Biol. Chem. *281*, 22917–22932.

Leontieva, O.V., Gudkov, A.V., and Blagosklonny, M.V. (2010). Weak p53 permits senescence during cell cycle arrest. Cell Cycle Georget. Tex *9*, 4323–4327.

Leontieva, O.V., Demidenko, Z.N., Gudkov, A.V., and Blagosklonny, M.V. (2011). Elimination of proliferating cells unmasks the shift from senescence to quiescence caused by rapamycin. PloS One *6*, e26126.

Levin, D.H., Kyner, D., and Acs, G. (1973). Protein initiation in eukaryotes: Formation and function of a ternary complex composed of a partially purified ribosomal factor, methionyl transfer RNAf, and guanosine triphosphate. Proc. Natl. Acad. Sci. *70*, 41–45.

Levy-Strumpf, N., Deiss, L.P., Berissi, H., and Kimchi, A. (1997). DAP-5, a novel homolog of eukaryotic translation initiation factor 4G isolated as a putative modulator of gamma interferon-induced programmed cell death. Mol. Cell. Biol. *17*, 1615–1625.

Lewis, S.M., and Holcik, M. (2007). For IRES trans-acting factors, it is all about location. Oncogene *27*, 1033–1035.

Li, P.F., Dietz, R., and von Harsdorf, R. (1999). p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J. *18*, 6027–6036.

Li, Y., Wang, Y., Kim, E., Beemiller, P., Wang, C.-Y., Swanson, J., You, M., and Guan, K.-L. (2007). Bnip3 Mediates the Hypoxia-induced Inhibition on Mammalian Target of Rapamycin by Interacting with Rheb. J. Biol. Chem. *282*, 35803–35813.

Lin, J.C., Hsu, M., and Tarn, W.Y. (2007). Cell stress modulates the function of splicing regulatory protein RBM4 in translation control. Proceedings of the National Academy of Sciences *104*, 2235.

Lin, T.A., Kong, X., Haystead, T.A., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J.C., Jr (1994). PHAS-I as a link between mitogen-activated protein kinase and translation initiation. Science *266*, 653–656.

Lindqvist, A., Rodriguez-Bravo, V., and Medema, R.H. (2009). The decision to enter mitosis: feedback and redundancy in the mitotic entry network. J. Cell Biol. *185*, 193–202.

Lindqvist, L., Imataka, H., and Pelletier, J. (2008). Cap-dependent eukaryotic initiation factor-mRNA interactions probed by cross-linking. RNA *14*, 960–969.

Ling, J., Morley, S.J., and Traugh, J.A. (2005). Inhibition of cap-dependent translation via phosphorylation of eIF4G by protein kinase Pak2. EMBO J. *24*, 4094–4105.

Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell *17*, 43–52.

Liu, B., Chen, Y., and St Clair, D.K. (2008). ROS and p53: a versatile partnership. Free Radic. Biol. Med. *44*, 1529–1535.

Liu, L., Cash, T.P., Jones, R.G., Keith, B., Thompson, C.B., and Simon, M.C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol. Cell *21*, 521–531.

Lockard, R.E., and Lane, C. (1978). Requirement for 7-methylguanosine in translation of globin mRNA in vivo. Nucleic Acids Res. *5*, 3237–3248.

Locker, N., Chamond, N., and Sargueil, B. (2011). A conserved structure within the HIV gag open reading frame that controls translation initiation directly recruits the 40S subunit and eIF3. Nucleic Acids Res. *39*, 2367–2377.

Lomakin, I.B., Hellen, C.U.T., and Pestova, T.V. (2000). Physical association of eukaryotic initiation factor 4G (eIF4G) with eIF4A strongly enhances binding of eIF4G to the internal ribosomal entry site of encephalomyocarditis virus and is required for internal initiation of translation. Molecular and Cellular Biology *20*, 6019–6029.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K., and Avruch, J. (2005). Rheb binds and regulates the MTOR kinase. Curr. Biol. CB *15*, 702–713.

López de Quinto, S., Lafuente, E., and Martínez-Salas, E. (2001). IRES interaction with translation initiation factors: functional characterization of novel RNA contacts with eIF3, eIF4B, and eIF4GII. RNA *7*, 1213–1226.

López de Quinto, S., Sáiz, M., de la Morena, D., Sobrino, F., and Martínez-Salas, E. (2002). IRES-driven translation is stimulated separately by the FMDV 3′-NCR and poly(A) sequences. Nucleic Acids Res. *30*, 4398–4405.

Lu, P.D., Harding, H.P., and Ron, D. (2004). Translation reinitiation at alternative open reading frames regulates gene expression in an integrated stress response. J. Cell Biol. *167*, 27–33.

Luukkonen, B.G., Tan, W., and Schwartz, S. (1995). Efficiency of reinitiation of translation on human immunodeficiency virus type 1 mRNAs is determined by the length of the upstream open reading frame and by intercistronic distance. Journal of Virology *69*, 4086–4094.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P.P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell *121*, 179–193.

Ma, X.M., Yoon, S.-O., Richardson, C.J., Jülich, K., and Blenis, J. (2008). SKAR links pre-mRNA splicing to MTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. Cell *133*, 303–313.

Maag, D., Fekete, C.A., Gryczynski, Z., and Lorsch, J.R. (2005). A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. Mol. Cell *17*, 265–275.

Macejak, D.G., and Sarnow, P. (1991). Internal initiation of translation mediated by the 5′leader of a cellular mRNA. Nature *353*, 90–94

MacLachlan, T.K., and El-Deiry, W.S. (2002). Apoptotic threshold is lowered by p53 transactivation of caspase-6. Proc. Natl. Acad. Sci. U. S. A. *99*, 9492–9497.

Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol. Cell. Biol. *15*, 4990–4997.

Majmundar, A.J., Wong, W.J., and Simon, M.C. (2010). Hypoxia-Inducible Factors and the Response to Hypoxic Stress. Mol. Cell *40*, 294–309.

Majumdar, R., Bandyopadhyay, A., and Maitra, U. (2003). Mammalian Translation Initiation Factor eIF1 Functions with eIF1A and eIF3 in the Formation of a Stable 40 S Preinitiation Complex. J. Biol. Chem. *278*, 6580–6587.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr, Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., and Tainsky, M.A. (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science *250*, 1233–1238.

Mamane, Y., Petroulakis, E., Martineau, Y., Sato, T.-A., Larsson, O., Rajasekhar, V.K., and Sonenberg, N. (2007). Epigenetic activation of a subset of mRNAs by eIF4E explains its effects on cell proliferation. PloS One *2*, e242.

Manalo, D.J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B.D., Ye, S.Q., Garcia, J.G.N., and Semenza, G.L. (2005). Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood *105*, 659–669.

Manfredi, J.J. (2010). The Mdm2–p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. Genes Dev. *24*, 1580–1589.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell *10*, 151–162.

Marcel, V., Perrier, S., Aoubala, M., Ageorges, S., Groves, M.J., Diot, A., Fernandes, K., Tauro, S., and Bourdon, J.-C. (2010b). Δ160p53 is a novel N-terminal p53 isoform encoded by Δ133p53 transcript. FEBS Lett. *584*, 4463–4468.

Marcel, V., Vijayakumar, V., Fernandez-Cuesta, L., Hafsi, H., Sagne, C., Hautefeuille, A., Olivier, M., and Hainaut, P. (2010a). p53 regulates the transcription of its Δ133p53 isoform through specific response elements contained within the TP53 P2 internal promoter. Oncogene *29*, 2691–2700.

Marciniak, S.J., Yun, C.Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H.P., and Ron, D. (2004). CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev. *18*, 3066–3077.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1997). Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell *89*, 951–961.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1999). Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol. Cell *3*, 707–716.

Marintchev, A., Edmonds, K.A., Marintcheva, B., Hendrickson, E., Oberer, M., Suzuki, C., Herdy, B., Sonenberg, N., and Wagner, G. (2009). Topology and Regulation of the Human eIF4A/4G/4H Helicase Complex in Translation Initiation. Cell *136*, 447–460.

Marissen, W.E., and Lloyd, R.E. (1998). Eukaryotic translation initiation factor 4G is targeted for proteolytic cleavage by caspase 3 during inhibition of translation in apoptotic cells. Mol. Cell. Biol. *18*, 7565–7574.

Martínez-Salas, E., Piñeiro, D., and Fernández, N. (2012). Alternative Mechanisms to Initiate Translation in Eukaryotic mRNAs. Comp. Funct. Genomics *2012*, 1–12.

Mashimo, T., Watabe, M., Hirota, S., Hosobe, S., Miura, K., Tegtmeyer, P.J., Rinker-Shaeffer, C.W., and Watabe, K. (1998). The expression of the KAI1 gene, a tumor metastasis suppressor, is directly activated by p53. Proc. Natl. Acad. Sci. U. S. A. *95*, 11307–11311.

Mathews, D.H., Sabina, J., Zuker, M., and Turner, D.H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. *288*, 911–940.

Matoba, S., Kang, J.-G., Patino, W.D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P.J., Bunz, F., and Hwang, P.M. (2006). p53 regulates mitochondrial respiration. Science *312*, 1650–1653.

Matsuda, D., and Dreher, T.W. (2006). Close spacing of AUG initiation codons confers dicistronic character on a eukaryotic mRNA. RNA *12*, 1338–1349.

Matsuo, H., Li, H., McGuire, A.M., Fletcher, C.M., Gingras, A.C., Sonenberg, N., and Wagner, G. (1997). Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. Nat. Struct. Biol. *4*, 717–724.

Mayer, C., Zhao, J., Yuan, X., and Grummt, I. (2004). MTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes Dev. *18*, 423–434.

Mazumder, B., Seshadri, V., Imataka, H., Sonenberg, N., and Fox, P.L. (2001). Translational Silencing of Ceruloplasmin Requires the Essential Elements of mRNA Circularization: Poly(A) Tail, Poly(A)-Binding Protein, and Eukaryotic Translation Initiation Factor 4G. Mol. Cell. Biol. *21*, 6440–6449.

Mazumder, B., Sampath, P., Seshadri, V., Maitra, R.K., DiCorleto, P.E., and Fox, P.L. (2003). Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. Cell *115*, 187–198.

McCullough, K.D., Martindale, J.L., Klotz, L.-O., Aw, T.-Y., and Holbrook, N.J. (2001). Gadd153 Sensitizes Cells to Endoplasmic Reticulum Stress by Down-Regulating Bcl2 and Perturbing the Cellular Redox State. Mol. Cell. Biol. *21*, 1249–1259.

McGivan, J.D., and Pastor-Anglada, M. (1994). Regulatory and molecular aspects of mammalian amino acid transport. Biochem. J. *299*, 321–0.

Melero, J.A., Stitt, D.T., Mangel, W.F., and Carroll, R.B. (1979). Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. Virology *93*, 466–480.

Melis, J.P.M., Hoogervorst, E.M., van Oostrom, C.T.M., Zwart, E., Breit, T.M., Pennings, J.L.A., de Vries, A., and van Steeg, H. (2011). Genotoxic exposure: novel cause of selection for a functional [Delta]N-p53 isoform. Oncogene *30*, 1764–1772.

Meng, Z., Jackson, N.L., Shcherbakov, O.D., Choi, H., and Blume, S.W. (2010). The Human IGF1R IRES Likely Operates Through a Shine–Dalgarno-Like Interaction With the G961 Loop (E-Site) of the 18S rRNA and Is Kinetically Modulated by a Naturally Polymorphic polyU Loop. J. Cell. Biochem. *110*, 531–544.

Méthot, N., Song, M.S., and Sonenberg, N. (1996). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. Mol. Cell. Biol. *16*, 5328–5334.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. Eur. J. Biochem. FEBS *267*, 6321–6330.

Michael, W.M., Eder, P.S., and Dreyfuss, G. (1997). The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. EMBO J. *16*, 3587–3598.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. Mol. Cell *11*, 577–590.

Mitchell, S.A., Spriggs, K.A., Bushell, M., Evans, J.R., Stoneley, M., Le Quesne, J.P.C., Spriggs, R.V., and Willis, A.E. (2005). Identification of a motif that mediates polypyrimidine tract-binding protein-dependent internal ribosome entry. Genes Dev. *19*, 1556–1571.

Mitchell, S.A., Spriggs, K.A., Coldwell, M.J., Jackson, R.J., and Willis, A.E. (2003). The APAF1 internal ribosome entry segment attains the correct structural conformation for function via interactions with PTB and unr. Mol. Cell *11*, 757–771.

Mitchell, S.F., Walker, S.E., Algire, M.A., Park, E.-H., Hinnebusch, A.G., and Lorsch, J.R. (2010). The 5′-7-Methylguanosine Cap on Eukaryotic mRNAs Serves Both to

Stimulate Canonical Translation Initiation and to Block an Alternative Pathway. Mol. Cell *39*, 950–962.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., and Reed, J.C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene *9*, 1799–1805.

Montanaro, L., Calienni, M., Bertoni, S., Rocchi, L., Sansone, P., Storci, G., Santini, D., Ceccarelli, C., Taffurelli, M., Carnicelli, D., et al. (2010). Novel Dyskerin-Mediated Mechanism of p53 Inactivation through Defective mRNA Translation. Cancer Res. *70*, 4767–4777.

Mori, Y., Nagse, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum. Mol. Genet. *1*, 229–233.

Moroni, M.C., Hickman, E.S., Lazzerini Denchi, E., Caprara, G., Colli, E., Cecconi, F., Müller, H., and Helin, K. (2001). APAF1 is a transcriptional target for E2F and p53. Nat. Cell Biol. *3*, 552–558.

Morris, D.R., and Geballe, A.P. (2000). Upstream open reading frames as regulators of mRNA translation. Molecular and Cellular Biology *20*, 8635–8642.

Mosner, J., Mummenbrauer, T., Bauer, C., Sczakiel, G., Grosse, F., and Deppert, W. (1995). Negative feedback regulation of wild-type p53 biosynthesis. EMBO J. *14*, 4442–4449.

Mounir, Z., Krishnamoorthy, J.L., Wang, S., Papadopoulou, B., Campbell, S., Muller, W.J., Hatzoglou, M., and Koromilas, A.E. (2011). Akt determines cell fate through inhibition of the PERK-eIF2α phosphorylation pathway. Sci. Signal. *4*, ra62.

Mount, S.M. (1982). A catalogue of splice junction sequences. Nucleic Acids Res. *10*, 459–472.

Mukhopadhyay, R., Jia, J., Arif, A., Ray, P.S., and Fox, P.L. (2009). The GAIT system: a gatekeeper of inflammatory gene expression. Trends Biochem. Sci. *34*, 324–331.

Nakajima, S., Hiramatsu, N., Hayakawa, K., Saito, Y., Kato, H., Huang, T., Yao, J., Paton, A.W., Paton, J.C., and Kitamura, M. (2011). Selective abrogation of BiP/GRP78 blunts activation of NF-κB through the ATF6 branch of the UPR: involvement of C/EBPβ and MTOR-dependent dephosphorylation of Akt. Mol. Cell. Biol. *31*, 1710–1718.

Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol. Cell *7*, 683–694.

Narayanan, C.S., and Dubnau, D. (1987). Demonstration of erythromycin-dependent stalling of ribosomes on the ermC leader transcript. J. Biol. Chem. *262*, 1766–1771.

Nejepinska, J., Malik, R., Moravec, M., and Svoboda, P. (2012). Deep Sequencing Reveals Complex Spurious Transcription from Transiently Transfected Plasmids. PLoS ONE *7*, e43283.

Nevins, T.A., Harder, Z.M., Korneluk, R.G., and Holcík, M. (2003). Distinct regulation of internal ribosome entry site-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G translation initiation factor family members eIF4GI and p97/DAP5/NAT1. J. Biol. Chem. *278*, 3572–3579.

Nishimori, H., Shiratsuchi, T., Urano, T., Kimura, Y., Kiyono, K., Tatsumi, K., Yoshida, S., Ono, M., Kuwano, M., Nakamura, Y., et al. (1997). A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. Oncogene *15*, 2145–2150.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002). ASK1 is essential for endoplasmic reticulum

stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev. *16*, 1345–1355.

Novoa, I., Zeng, H., Harding, H.P., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. J. Cell Biol. *153*, 1011–1022.

Obrig, T.G., Culp, W.J., McKeehan, W.L., and Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J. Biol. Chem. *246*, 174–181.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053–1058.

Oh, W.J., and Jacinto, E. (2011). MTOR complex 2 signaling and functions. Cell Cycle *10*, 2305–2316.

Ohoka, N., Yoshii, S., Hattori, T., Onozaki, K., and Hayashi, H. (2005). TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. EMBO J. *24*, 1243–1255.

Olivares-Illana, V., and F\aahraeus, R. (2010). p53 isoforms gain functions. Oncogene *29*, 5113–5119.

Oliver, F.J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M.C., de Murcia, G., and Murcia, J.M. (1998). Importance of Poly(ADP-ribose) Polymerase and Its Cleavage in Apoptosis: LESSON FROM AN UNCLEAVABLE MUTANT. J. Biol. Chem. *273*, 33533–33539.

Olschwang, S., Laurent-Puig, P., Groden, J., White, R., and Thomas, G. (1993). Germ-line mutations in the first 14 exons of the adenomatous polyposis coli (APC) gene. Am. J. Hum. Genet. *52*, 273.

Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J., and Yonezawa, K. (2004). Dissociation of raptor from MTOR is a mechanism of rapamycin-induced inhibition of MTOR function. Genes Cells *9*, 359–366.

Ozcan, U., Ozcan, L., Yilmaz, E., Düvel, K., Sahin, M., Manning, B.D., and Hotamisligil, G.S. (2008). Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis. Mol. Cell *29*, 541–551.

Pacheco, A., Reigadas, S., and Martinez-Salas, E. (2008). Riboproteomic analysis of polypeptides interacting with the internal ribosome-entry site element of foot-and-mouth disease viral RNA. Proteomics *8*, 4782–4790.

Pan, P., and van Breukelen, F. (2011). Preference of IRES-mediated initiation of translation during hibernation in golden-mantled ground squirrels, Spermophilus lateralis. AJP: Regulatory, Integrative and Comparative Physiology *301*, R370–R377.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D., and Rotter, V. (1984). Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature *312*, 649–651.

Park, E.-H., Walker, S.E., Lee, J.M., Rothenburg, S., Lorsch, J.R., and Hinnebusch, A.G. (2011). Multiple elements in the eIF4G1 N-terminus promote assembly of eIF4G1[bull]PABP mRNPs in vivo. EMBO J *30*, 302–316.

Passmore, L.A., Schmeing, T.M., Maag, D., Applefield, D.J., Acker, M.G., Algire, M.A., Lorsch, J.R., and Ramakrishnan, V. (2007). The Eukaryotic Translation Initiation

Factors eIF1 and eIF1A Induce an Open Conformation of the 40S Ribosome. Mol. Cell *26*, 41–50.

Patarroyo, M., Tryggvason, K., and Virtanen, I. (2002). Laminin isoforms in tumor invasion, angiogenesis and metastasis. Semin. Cancer Biol. *12*, 197–207.

Patel, S., Doble, B., and Woodgett, J.R. (2004). Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged sword? Biochem. Soc. Trans. *32*, 803–808.

Pause, A., Belsham, G.J., Gingras, A.-C., Donze, O., Lin, T.-A., Lawrence, J.C., and Sonenberg, N. (1994). Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature *371*, 762–767.

Pavitt, G.D. (2005). eIF2B, a mediator of general and gene-specific translational control. Biochemical Society Transactions *33*, 1487.

Pawlus, M.R., and Hu, C.-J. (2013). Enhanceosomes as integrators of hypoxia inducible factor (HIF) and other transcription factors in the hypoxic transcriptional response. Cell. Signal. *25*, 1895–1903.

Peabody, D.S. (1989). Translation initiation at non-AUG triplets in mammalian cells. Journal of Biological Chemistry *264*, 5031–5035.

Pearce, L.R., Huang, X., Boudeau, J., Pawłowski, R., Wullschleger, S., Deak, M., Ibrahim, A.F.M., Gourlay, R., Magnuson, M.A., and Alessi, D.R. (2007). Identification of Protor as a novel Rictor-binding component of MTOR complex-2. Biochem. J. *405*, 513–522.

Pearce, L.R., Sommer, E.M., Sakamoto, K., Wullschleger, S., and Alessi, D.R. (2011). Protor-1 is required for efficient MTORC2-mediated activation of SGK1 in the kidney. Biochem. J. *436*, 169–179.

Pelletier, J., and Sonenberg, N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature *334*, 320–325.

Pestova, T.V., and Kolupaeva, V.G. (2002). The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. Genes Dev. *16*, 2906–2922.

Pestova, T.V., Borukhov, S.I., and Hellen, C.U.T. (1998). Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature *394*, 854–859.

Pestova, T.V., de Breyne, S., Pisarev, A.V., Abaeva, I.S., and Hellen, C.U. (2008). eIF2-dependent and eIF2-independent modes of initiation on the CSFV IRES: a common role of domain II. EMBO J. *27*, 1060–1072.

Pestova, T.V., Hellen, C.U., and Wimmer, E. (1991). Translation of poliovirus RNA: role of an essential cis-acting oligopyrimidine element within the 5' nontranslated region and involvement of a cellular 57-kilodalton protein. J. Virol. *65*, 6194–6204.

Pestova, T.V., Lomakin, I.B., Lee, J.H., Choi, S.K., Dever, T.E., and Hellen, C.U. (2000). The joining of ribosomal subunits in eukaryotes requires eIF5B. Nature *403*, 332–335.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., and Sabatini, D.M. (2009). DEPTOR is an MTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell *137*, 873–886.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P., and Olivier, M. (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum. Mutat. *28*, 622–629.

Petz, M., Kozina, D., Huber, H., Siwiec, T., Seipelt, J., Sommergruber, W., and Mikulits, W. (2007). The leader region of Laminin B1 mRNA confers cap-independent translation. Nucleic Acids Research *35*, 2473–2482.

Petz, M., Them, N., Huber, H., Beug, H., and Mikulits, W. (2012). La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. Nucleic Acids Res. *40*, 290–302.

Petz, M., Them, N., Huber, H., Beug, H., and Mikulits, W. (2012). La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. Nucleic Acids Res. *40*, 290–302.

Pickering, B.M., Mitchell, S.A., Evans, J.R., and Willis, A.E. (2003). Polypyrimidine tract binding protein and poly r(C) binding protein 1 interact with the BAG-1 IRES and stimulate its activity in vitro and in vivo. Nucleic Acids Res. *31*, 639–646.

Pickering, B.M., Mitchell, S.A., Spriggs, K.A., Stoneley, M., and Willis, A.E. (2004). BAG1 Internal Ribosome Entry Segment Activity Is Promoted by Structural Changes Mediated by Poly(rC) Binding Protein 1 and Recruitment of Polypyrimidine Tract Binding Protein 1. Mol. Cell. Biol. *24*, 5595–5605.

Pinkstaff, J.K., Chappell, S.A., Mauro, V.P., Edelman, G.M., and Krushel, L.A. (2001). Internal initiation of translation of five dendritically localized neuronal mRNAs. Proc. Natl. Acad. Sci. *98*, 2770–2775.

Pisarev, A.V., Kolupaeva, V.G., Pisareva, V.P., Merrick, W.C., Hellen, C.U.T., and Pestova, T.V. (2006). Specific functional interactions of nucleotides at key -3 and +4 positions flanking the initiation codon with components of the mammalian 48S translation initiation complex. Genes Dev. *20*, 624–636.

Piñol-Roma, S. (1997). HnRNP proteins and the nuclear export of mRNA. Semin. Cell Dev. Biol. *8*, 57–63.

Pluquet, O., Qu, L.-K., Baltzis, D., and Koromilas, A.E. (2005). Endoplasmic reticulum stress accelerates p53 degradation by the cooperative actions of Hdm2 and glycogen synthase kinase 3beta. Mol. Cell. Biol. *25*, 9392–9405.

Pópulo, H., Lopes, J.M., and Soares, P. (2012). The MTOR Signalling Pathway in Human Cancer. Int. J. Mol. Sci. *13*, 1886–1918.

Portt, L., Norman, G., Clapp, C., Greenwood, M., and Greenwood, M.T. (2011). Anti-apoptosis and cell survival: A review. Biochim. Biophys. Acta BBA - Mol. Cell Res. *1813*, 238–259.

Potter, C.J., Pedraza, L.G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat. Cell Biol. *4*, 658–665.

Poulin, F., Gingras, A.C., Olsen, H., Chevalier, S., and Sonenberg, N. (1998). 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J. Biol. Chem. *273*, 14002–14007.

Powell, D.J., Hrstka, R., Candeias, M., Bourougaa, K., Vojtesek, B., and Fåhraeus, R. (2008). Stress-dependent changes in the properties of p53 complexes by the alternative translation product p53/47. Cell Cycle Georget. Tex *7*, 950–959.

Powley, I.R., Kondrashov, A., Young, L.A., Dobbyn, H.C., Hill, K., Cannell, I.G., Stoneley, M., Kong, Y.-W., Cotes, J.A., Smith, G.C.M., et al. (2009). Translational reprogramming following UVB irradiation is mediated by DNA-PKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes. Genes Dev. *23*, 1207–1220.

Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992). APC mutations occur early during colorectal tumorigenesis. Nature *359*, 235–237.

Pöyry, T.A.A., Kaminski, A., and Jackson, R.J. (2004). What determines whether mammalian ribosomes resume scanning after translation of a short upstream open reading frame? Genes Dev. *18*, 62–75.

Poyry, T.A.A., Kaminski, A., Connell, E.J., Fraser, C.S., and Jackson, R.J. (2007). The mechanism of an exceptional case of reinitiation after translation of a long ORF reveals why such events do not generally occur in mammalian mRNA translation. Genes & Development *21*, 3149–3162.

Proud, C.G. (2005). eIF2 and the control of cell physiology. Protein Synth. Heal. Dis. *16*, 3–12.

Proud, C.G., Wang, X., Patel, J.V., Campbell, L.E., Kleijn, M., Li, W., and Browne, G.J. (2001). Interplay between insulin and nutrients in the regulation of translation factors. Biochem. Soc. Trans. *29*, 541–547.

Pyronnet, S., Dostie, J., and Sonenberg, N. (2001). Suppression of cap-dependent translation in mitosis. Genes Dev. *15*, 2083–2093.

Pyronnet, S., Pradayrol, L., and Sonenberg, N. (2000). A cell cycle-dependent internal ribosome entry site. Mol. Cell *5*, 607–616.

Qin, X., and Sarnow, P. (2004). Preferential translation of internal ribosome entry site-containing mRNAs during the mitotic cycle in mammalian cells. J. Biol. Chem. *279*, 13721–13728.

Qin, L., Wang, Z., Tao, L., and Wang, Y. (2010). ER stress negatively regulates AKT/TSC/MTOR pathway to enhance autophagy. Autophagy *6*, 239–247.

Qu, L., Huang, S., Baltzis, D., Rivas-Estilla, A.-M., Pluquet, O., Hatzoglou, M., Koumenis, C., Taya, Y., Yoshimura, A., and Koromilas, A.E. (2004). Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. Genes Dev. *18*, 261–277.

Ramirez-Valle, F., Badura, M.L., Braunstein, S., Narasimhan, M., and Schneider, R.J. (2010). Mitotic Raptor Promotes MTORC1 Activity, G2/M Cell Cycle Progression, and Internal Ribosome Entry Site-Mediated mRNA Translation. Mol. Cell. Biol. *30*, 3151–3164.

Ramírez-Valle, F., Braunstein, S., Zavadil, J., Formenti, S.C., and Schneider, R.J. (2008). eIF4GI links nutrient sensing by MTOR to cell proliferation and inhibition of autophagy. J. Cell Biol. *181*, 293–307.

Raught, B., Gingras, A.C., Gygi, S.P., Imataka, H., Morino, S., Gradi, A., Aebersold, R., and Sonenberg, N. (2000). Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. EMBO J. *19*, 434–444.

Raught, B., Peiretti, F., Gingras, A.-C., Livingstone, M., Shahbazian, D., Mayeur, G.L., Polakiewicz, R.D., Sonenberg, N., and Hershey, J.W.B. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. EMBO J. *23*, 1761–1769.

Ray, P.S., Grover, R., and Das, S. (2006). Two internal ribosome entry sites mediate the translation of p53 isoforms. EMBO Rep.

Reed, R. (1989). The organization of 3' splice-site sequences in mammalian introns. Genes Dev. *3*, 2113–2123.

Reiling, J.H., and Hafen, E. (2004). The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. Genes Dev. *18*, 2879–2892.

Reynolds, J.E., Kaminski, A., Kettinen, H.J., Grace, K., Clarke, B.E., Carroll, A.R., Rowlands, D.J., and Jackson, R.J. (1995). Unique features of internal initiation of hepatitis C virus RNA translation. EMBO J. *14*, 6010–6020.

Richardson, C.J., Bröenstrup, M., Fingar, D.C., Jülich, K., Ballif, B.A., Gygi, S., and Blenis, J. (2004). SKAR is a specific target of S6 kinase 1 in cell growth control. Curr. Biol. CB *14*, 1540–1549.

Richter, N.J., Rogers, G.W., Hensold, J.O., and Merrick, W.C. (1999). Further Biochemical and Kinetic Characterization of Human Eukaryotic Initiation Factor 4H. J. Biol. Chem. *274*, 35415–35424.

Rijnbrand, R., van der Straaten, T., van Rijn, P.A., Spaan, W.J., and Bredenbeek, P.J. (1997). Internal entry of ribosomes is directed by the 5' noncoding region of classical swine fever virus and is dependent on the presence of an RNA pseudoknot upstream of the initiation codon. J. Virol. *71*, 451–457.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat. Rev. Mol. Cell Biol. *9*, 402–412.

Rivlin, N., Brosh, R., Oren, M., and Rotter, V. (2011). Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer *2*, 466–474.

Robles, A.I., Bemmels, N.A., Foraker, A.B., and Harris, C.C. (2001). APAF1 is a transcriptional target of p53 in DNA damage-induced apoptosis. Cancer Res. *61*, 6660–6664.

Roccio, M., Bos, J.L., and Zwartkruis, F.J.T. (2006). Regulation of the small GTPase Rheb by amino acids. Oncogene *25*, 657–664.

Rodríguez Pulido, M., Serrano, P., Sáiz, M., and Martínez-Salas, E. (2007). Foot-and-mouth disease virus infection induces proteolytic cleavage of PTB, eIF3a,b, and PABP RNA-binding proteins. Virology *364*, 466–474.

Rogers, G.W., Jr, Richter, N.J., and Merrick, W.C. (1999). Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. J. Biol. Chem. *274*, 12236–12244.

Rogers, G.W., Jr, Richter, N.J., Lima, W.F., and Merrick, W.C. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. J. Biol. Chem. *276*, 30914–30922.

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol *8*, 519–529.

Roos, W.P., and Kaina, B. (2006). DNA damage-induced cell death by apoptosis. Trends Mol. Med. *12*, 440–450.

Rosen, N., and She, Q.-B. (2006). AKT and cancer--is it all MTOR? Cancer Cell *10*, 254–256.

Rosenwald, I.B., Chen, J.J., Wang, S., Savas, L., London, I.M., and Pullman, J. (1999). Upregulation of protein synthesis initiation factor eIF-4E is an early event during colon carcinogenesis. Oncogene *18*, 2507–2517.

Rosenwald, I.B., Wang, S., Savas, L., Woda, B., and Pullman, J. (2003). Expression of translation initiation factor eIF-2alpha is increased in benign and malignant melanocytic and colonic epithelial neoplasms. Cancer *98*, 1080–1088.

Rotter, V. (1983). p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells. Proc. Natl. Acad. Sci. *80*, 2613–2617.

Rotter, V., Witte, O.N., Coffman, R., and Baltimore, D. (1980). Abelson murine leukemia virus-induced tumors elicit antibodies against a host cell protein, P50. J. Virol. *36*, 547–555.

Roux, P.P., Ballif, B.A., Anjum, R., Gygi, S.P., and Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc. Natl. Acad. Sci. U. S. A. *101*, 13489–13494.

Ruggero, D. (2012). Translational Control in Cancer Etiology. Cold Spring Harb. Perspect. Biol. *5*, a012336–a012336.

Ruggero, D. (2009). The role of Myc-induced protein synthesis in cancer. Cancer Res. *69*, 8839–8843.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35–43.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and Abraham, R.T. (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem. *270*, 815–822.

Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E., and Chumakov, P.M. (2005). The antioxidant function of the p53 tumor suppressor. Nat. Med. *11*, 1306–1313.

Sampath, P., Mazumder, B., Seshadri, V., Gerber, C.A., Chavatte, L., Kinter, M., Ting, S.M., Dignam, J.D., Kim, S., and Driscoll, D.M. (2004). Noncanonical function of glutamyl-prolyl-tRNA synthetase: gene-specific silencing of translation. Cell *119*, 195–208.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 Is an Insulin-Regulated Inhibitor of the MTORC1 Protein Kinase. Mol. Cell *25*, 903–915.

Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to MTORC1. Science *320*, 1496–1501.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A.L., Nada, S., and Sabatini, D.M. (2010). Ragulator-Rag Complex Targets MTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids. Cell *141*, 290–303.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of MTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol. *14*, 1296–1302.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.-H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged Rapamycin Treatment Inhibits MTORC2 Assembly and Akt/PKB. Mol. Cell *22*, 159–168.

Sarnow, P. (1989). Translation of glucose-regulated protein 78/immunoglobulin heavy-chain binding protein mRNA is increased in poliovirus-infected cells at a time when cap-dependent translation of cellular mRNAs is inhibited. Proc. Natl. Acad. Sci. *86*, 5795–5799.

Sax, J.K., Fei, P., Murphy, M.E., Bernhard, E., Korsmeyer, S.J., and El-Deiry, W.S. (2002). BID regulation by p53 contributes to chemosensitivity. Nat. Cell Biol. *4*, 842–849.

Schewe, D.M., and Aguirre-Ghiso, J.A. (2008). ATF6alpha-Rheb-MTOR signaling promotes survival of dormant tumor cells in vivo. Proc. Natl. Acad. Sci. U. S. A. *105*, 10519–10524.

Schmitt, C.A., Fridman, J.S., Yang, M., Lee, S., Baranov, E., Hoffman, R.M., and Lowe, S.W. (2002). A Senescence Program Controlled by p53 and p16INK4a Contributes to the Outcome of Cancer Therapy. Cell *109*, 335–346.

Schneider, R.J., and Mohr, I. (2003). Translation initiation and viral tricks. Trends Biochem. Sci. *28*, 130–136.

Schneider, R.J., and Shenk, T. (1987). Impact of Virus Infection on Host Cell Protein Synthesis. Annu. Rev. Biochem. *56*, 317–332.

Schwab, M.S., Kim, S.H., Terada, N., Edfjäll, C., Kozma, S.C., Thomas, G., and Maller, J.L. (1999). p70(S6K) controls selective mRNA translation during oocyte maturation and early embryogenesis in Xenopus laevis. Mol. Cell. Biol. *19*, 2485–2494.

Schwartzenberg-Bar-Yoseph, F., Armoni, M., and Karnieli, E. (2004). The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res. *64*, 2627–2633.

Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P., and Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J. Biol. Chem. *271*, 32529–32537.

Semler, B., and Waterman, M. (2008). IRES-mediated pathways to polysomes: nuclear versus cytoplasmic routes. Trends Microbiol. *16*, 1–5.

Senapathy, P., Shapiro, M.B., and Harris, N.L. (1990). Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project. In Methods in Enzymology, (Academic Press), pp. 252–278.

Sengupta, S., Peterson, T.R., and Sabatini, D.M. (2010). Regulation of the MTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress. Mol. Cell *40*, 310–322.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras Provokes Premature Cell Senescence Associated with Accumulation of p53 and p16INK4a. Cell *88*, 593–602.

Serrano, P., Ramajo, J., and Martínez-Salas, E. (2009). Rescue of internal initiation of translation by RNA complementation provides evidence for a distribution of functions between individual IRES domains. Virology *388*, 221–229.

Shahbazian, D., Roux, P.P., Mieulet, V., Cohen, M.S., Raught, B., Taunton, J., Hershey, J.W.B., Blenis, J., Pende, M., and Sonenberg, N. (2006). The MTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO J. *25*, 2781–2791.

Sharathchandra, A., Lal, R., Khan, D., and Das, S. (2012). Annexin A2 and PSF proteins interact with p53 IRES and regulate translation of p53 mRNA. RNA Biol. *9*, 1429–1439.

Shaughnessy, J.D., Jr, Jenkins, N.A., and Copeland, N.G. (1997). cDNA cloning, expression analysis, and chromosomal localization of a gene with high homology to wheat eIF-(iso)4F and mammalian eIF-4G. Genomics *39*, 192–197.

Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A., and Rotter, V. (1990). Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. Mol. Cell. Biol. *10*, 6565–6577.

She, Q.-B., Halilovic, E., Ye, Q., Zhen, W., Shirasawa, S., Sasazuki, T., Solit, D.B., and Rosen, N. (2010). 4E-BP1 Is a Key Effector of the Oncogenic Activation of the AKT and ERK Signaling Pathways that Integrates Their Function in Tumors. Cancer Cell *18*, 39–51.

Sherrill, K.W., Byrd, M.P., Van Eden, M.E., and Lloyd, R.E. (2004). BCL-2 translation is mediated via internal ribosome entry during cell stress. J. Biol. Chem. *279*, 29066–29074.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S.C. (1998). Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J. *17*, 6649–6659.

Shor, B., Wu, J., Shakey, Q., Toral-Barza, L., Shi, C., Follettie, M., and Yu, K. (2010). Requirement of the MTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. J. Biol. Chem. *285*, 15380–15392.

Shuman, S. (2001). The mRNA Capping Apparatus as Drug Target and Guide to Eukaryotic Phylogeny. Cold Spring Harb. Symp. Quant. Biol. *66*, 301–312.

Silvera, D., Arju, R., Darvishian, F., Levine, P.H., Zolfaghari, L., Goldberg, J., Hochman, T., Formenti, S.C., and Schneider, R.J. (2009). Essential role for eIF4GI overexpression in the pathogenesis of inflammatory breast cancer. Nat. Cell Biol. *11*, 903–908.

Silvera, D., Formenti, S.C., and Schneider, R.J. (2010). Translational control in cancer. Nat. Rev. Cancer *10*, 254–266.

Skabkin, M.A., Skabkina, O.V., Dhote, V., Komar, A.A., Hellen, C.U.T., and Pestova, T.V. (2010). Activities of Ligatin and MCT-1/DENR in eukaryotic translation initiation and ribosomal recycling. Genes Dev. *24*, 1787–1801.

Skabkin, M.A., Skabkina, O.V., Hellen, C.U.T., and Pestova, T.V. (2013). Reinitiation and Other Unconventional Posttermination Events during Eukaryotic Translation. Mol. Cell *51*, 249–264.

Smith, A.E., Smith, R., and Paucha, E. (1979). Characterization of different tumor antigens present in cells transformed by simian virus 40. Cell *18*, 335–346.

Smith, A.J., Stern, H.S., Penner, M., Hay, K., Mitri, A., Bapat, B.V., and Gallinger, S. (1994). Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer Res. *54*, 5527–5530.

Spirio, L., Olschwang, S., Groden, J., Robertson, M., Samowitz, W., Joslyn, G., Gelbert, L., Thliveris, A., Carlson, M., Otterud, B., et al. (1993). Alleles of the APC gene: an attenuated form of familial polyposis. Cell *75*, 951–957.

Su, L.K., Barnes, C.J., Yao, W., Qi, Y., Lynch, P.M., and Steinbach, G. (2000). Inactivation of germline mutant APC alleles by attenuated somatic mutations: a molecular genetic mechanism for attenuated familial adenomatous polyposis. Am. J. Hum. Genet. *67*, 582–590.

Szamecz, B., Rutkai, E., Cuchalová, L., Munzarová, V., Herrmannová, A., Nielsen, K.H., Burela, L., Hinnebusch, A.G., and Valásek, L. (2008). eIF3a cooperates with sequences 5' of uORF1 to promote resumption of scanning by post-termination ribosomes for reinitiation on GCN4 mRNA. Genes Dev. *22*, 2414–2425.

Slatter, T.L., Hung, N., Campbell, H., Rubio, C., Mehta, R., Renshaw, P., Williams, G., Wilson, M., Engelmann, A., Jeffs, A., et al. (2011). Hyperproliferation, cancer, and inflammation in mice expressing a Δ133p53-like isoform. Blood *117*, 5166–5177.

Smith, E.M., Finn, S.G., Tee, A.R., Browne, G.J., and Proud, C.G. (2005). The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. J. Biol. Chem. *280*, 18717–18727.

Smith, M.L., Chen, I.T., Zhan, Q., Bae, I., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M., and Fornace, A.J., Jr (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science *266*, 1376–1380.

Sonenberg, N., and Hinnebusch, A.G. (2007). New Modes of Translational Control in Development, Behavior, and Disease. Mol. Cell *28*, 721–729.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. Cell *136*, 731–745.

Sonna, L.A., Fujita, J., Gaffin, S.L., and Lilly, C.M. (2002). Invited review: effects of heat and cold stress on mammalian gene expression. J. Appl. Physiol. *92*, 1725–1742.

Spriggs, K.A., Cobbold, L.C., Jopling, C.L., Cooper, R.E., Wilson, L.A., Stoneley, M., Coldwell, M.J., Poncet, D., Shen, Y.C., Morley, S.J., et al. (2009). Canonical initiation factor requirements of the Myc family of internal ribosome entry segments. Molecular and Cellular Biology *29*, 1565.

Spriggs, K., Stoneley, M., Bushell, M., and Willis, A. (2008). Re-programming of translation following cell stress allows IRES-mediated translation to predominate. Biol. Cell *100*, 27–38.

Stewart, N., Hicks, G.G., Paraskevas, F., and Mowat, M. (1995). Evidence for a second cell cycle block at G2/M by p53. Oncogene *10*, 109–115.

Stolovich, M., Tang, H., Hornstein, E., Levy, G., Cohen, R., Bae, S.S., Birnbaum, M.J., and Meyuhas, O. (2002). Transduction of growth or mitogenic signals into translational activation of TOP mRNAs is fully reliant on the phosphatidylinositol 3-kinase-mediated pathway but requires neither S6K1 nor rpS6 phosphorylation. Mol. Cell. Biol. *22*, 8101–8113.

Stoneley, M., Paulin, F.E., Le Quesne, J.P., Chappell, S.A., and Willis, A.E. (1998). C-Myc 5' untranslated region contains an internal ribosome entry segment. Oncogene *16*, 423–428.

Stoneley, M., Chappell, S.A., Jopling, C.L., Dickens, M., MacFarlane, M., and Willis, A.E. (2000a). c-Myc protein synthesis is initiated from the internal ribosome entry segment during apoptosis. Mol. Cell. Biol. *20*, 1162–1169.

Stoneley, M., Subkhankulova, T., Le Quesne, J.P., Coldwell, M.J., Jopling, C.L., Belsham, G.J., and Willis, A.E. (2000b). Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment. Nucleic Acids Res. *28*, 687–694.

Strauss, B.S. (2000). Role in tumorigenesis of silent mutations in the TP53 gene. Mutat. Res. Mol. Mech. Mutagen. *457*, 93–104.

Summerton, J. (1999). Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim. Biophys. Acta *1489*, 141–158.

Suzuki, C., Garces, R.G., Edmonds, K.A., Hiller, S., Hyberts, S.G., Marintchev, A., and Wagner, G. (2008). PDCD4 inhibits translation initiation by binding to eIF4A using both its MA3 domains. Proc. Natl. Acad. Sci. *105*, 3274–3279.

Suzuki, K., Dashzeveg, N., Lu, Z.-G., Taira, N., Miki, Y., and Yoshida, K. (2012). Programmed cell death 6, a novel p53-responsive gene, targets to the nucleus in the apoptotic response to DNA damage. Cancer Sci. *103*, 1788–1794.

Svitkin, Y.V., Pause, A., Haghighat, A., Pyronnet, S., Witherell, G., Belsham, G.J., and Sonenberg, N. (2001). The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. RNA New York N *7*, 382–394.

Svitkin, Y.V., Herdy, B., Costa-Mattioli, M., Gingras, A.C., Raught, B., and Sonenberg, N. (2005). Eukaryotic translation initiation factor 4E availability controls the switch between cap-dependent and internal ribosomal entry site-mediated translation. Mol. Cell. Biol. *25*, 10556.

Svitkin, Y.V., Evdokimova, V.M., Brasey, A., Pestova, T.V., Fantus, D., Yanagiya, A., Imataka, H., Skabkin, M.A., Ovchinnikov, L.P., Merrick, W.C., et al. (2009). General RNA-binding proteins have a function in poly(A)-binding protein-dependent translation. EMBO J *28*, 58–68.

Tabas, I., and Ron, D. (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat. Cell Biol. *13*, 184–190.

Tahiri-Alaoui, A., Smith, L.P., Baigent, S., Kgosana, L., Petherbridge, L.J., Lambeth, L.S., James, W., and Nair, V. (2009). Identification of an Intercistronic Internal Ribosome Entry Site in a Marek's Disease Virus Immediate-Early Gene. J. Virol. *83*, 5846–5853.

Takagi, M., Absalon, M.J., McLure, K.G., and Kastan, M.B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell *123*, 49–63.

Tan, C.Y., and Hagen, T. (2013). Post-translational regulation of MTOR complex 1 in hypoxia and reoxygenation. Cell. Signal. *25*, 1235–1244.

Tan, T., and Chu, G. (2002). p53 Binds and Activates the Xeroderma Pigmentosum DDB2 Gene in Humans but Not Mice. Mol. Cell. Biol. *22*, 3247–3254.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D., Avruch, J., and Meyuhas, O. (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol. Cell. Biol. *21*, 8671–8683.

Tate, W.P., Poole, E.S., and Mannering, S.A. (1996). Hidden infidelities of the translational stop signal. Prog. Nucleic Acid Res. Mol. Biol. *52*, 293–335.

Tee, A.R., and Proud, C.G. (2002). Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. Mol. Cell. Biol. *22*, 1674–1683.

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., and Blenis, J. (2003). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control MTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr. Biol. CB *13*, 1259–1268.

Tee, A.R., Tee, J.A., and Blenis, J. (2004). Characterizing the interaction of the mammalian eIF4E-related protein 4EHP with 4E-BP1. FEBS Letters *564*, 58–62.

Teodoro, J.G., Parker, A.E., Zhu, X., and Green, M.R. (2006). p53-mediated inhibition of angiogenesis through up-regulation of a collagen prolyl hydroxylase. Science *313*, 968–971.

Thakor, N., and Holcik, M. (2011). IRES-mediated translation of cellular messenger RNA operates in eIF2 - independent manner during stress. Nucleic Acids Res. *40*, 541–552.

Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R., and Dawson, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2 (+)-ATPase. Proc. Natl. Acad. Sci. *87*, 2466–2470.

Thedieck, K., Polak, P., Kim, M.L., Molle, K.D., Cohen, A., Jenö, P., Arrieumerlou, C., and Hall, M.N. (2007). PRAS40 and PRR5-like protein are new MTOR interactors that regulate apoptosis. PloS One *2*, e1217.

Theil, E.C. (1993). The IRE (iron regulatory element) family: structures which regulate mRNA translation or stability. Biofactors *4*, 87–93.

Thiery, J.P., Acloque, H., Huang, R.Y.J., and Nieto, M.A. (2009). Epithelial-mesenchymal transitions in development and disease. Cell *139*, 871–890.

Thoma, C., Bergamini, G., Galy, B., Hundsdoerfer, P., and Hentze, M.W. (2004). Enhancement of IRES-mediated translation of the c-myc and BiP mRNAs by the poly(A) tail is independent of intact eIF4G and PABP. Mol. Cell *15*, 925–935.

Thomas, G.V., Tran, C., Mellinghoff, I.K., Welsbie, D.S., Chan, E., Fueger, B., Czernin, J., and Sawyers, C.L. (2006). Hypoxia-inducible factor determines sensitivity to inhibitors of MTOR in kidney cancer. Nat Med *12*, 122–127.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive Mammalian Target of Rapamycin Inhibitor Reveals Rapamycin-resistant Functions of MTORC1. J. Biol. Chem. *284*, 8023–8032.

Thoreen, C.C., Chantranupong, L., Keys, H.R., Wang, T., Gray, N.S., and Sabatini, D.M. (2012). A unifying model for MTORC1-mediated regulation of mRNA translation. Nature *485*, 109–113.

Tokunaga, E., Oki, E., Egashira, A., Sadanaga, N., Morita, M., Kakeji, Y., and Maehara, Y. (2008). Deregulation of the Akt pathway in human cancer. Curr. Cancer Drug Targets *8*, 27–36.

Topisirovic, I., Guzman, M.L., McConnell, M.J., Licht, J.D., Culjkovic, B., Neering, S.J., Jordan, C.T., and Borden, K.L.B. (2003). Aberrant eukaryotic translation initiation factor 4E-dependent mRNA transport impedes hematopoietic differentiation and contributes to leukemogenesis. Mol. Cell. Biol. *23*, 8992–9002.

Tsukahara, T., Haniu, H., and Matsuda, Y. (2013). PTB-Associated Splicing Factor (PSF) Is a PPARγ-Binding Protein and Growth Regulator of Colon Cancer Cells. PLoS ONE *8*, e58749.

Tzatsos, A., and Kandror, K.V. (2006). Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent MTOR-mediated insulin receptor substrate 1 phosphorylation. Mol. Cell. Biol. *26*, 63–76.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J., et al. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature *431*, 200–205.

Unbehaun, A., Borukhov, S.I., Hellen, C.U.T., and Pestova, T.V. (2004). Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. Genes Dev. *18*, 3078–3093.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H.P., and Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science *287*, 664–666.

Van Breukelen, F., and Martin, S.L. (2001). Translational initiation is uncoupled from elongation at 18 degrees C during mammalian hibernation. Am. J. Physiol. Regul. Integr. Comp. Physiol. *281*, R1374–1379.

Van Oordt, W. van der H., Diaz-Meco, M.T., Lozano, J., Krainer, A.R., Moscat, J., and Cáceres, J.F. (2000). The MKK3/6-p38–signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. The Journal of Cell Biology *149*, 307–316.

Van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Reuser, A., Sampson, J., Halley, D., and van der Sluijs, P. (1998). Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. Hum. Mol. Genet. *7*, 1053–1057.

Vander Haar, E., Lee, S.-I., Bandhakavi, S., Griffin, T.J., and Kim, D.-H. (2007). Insulin signalling to MTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol. *9*, 316–323.

Vasen, H.F.A., Möslein, G., Alonso, A., Aretz, S., Bernstein, I., Bertario, L., Blanco, I., Bülow, S., Burn, J., Capella, G., et al. (2008). Guidelines for the clinical management of familial adenomatous polyposis (FAP). Gut *57*, 704–713

Vattem, K.M., and Wek, R.C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. *101*, 11269–11274.

Vazquez-Pianzola, P., Hernández, G., Suter, B., and Rivera-Pomar, R. (2006). Different modes of translation for hid, grim and sickle mRNAs in Drosophila. Cell Death and Differentiation *14*, 286–295.

Vazquez-Pianzola, P., Urlaub, H., and Rivera-Pomar, R. (2005). Proteomic analysis of reaper 5' untranslated region-interacting factors isolated by tobramycin affinity-selection reveals a role for La antigen in reaper mRNA translation. Proteomics *5*, 1645–1655.

Vega-Rubin-de-Celis, S., Abdallah, Z., Kinch, L., Grishin, N.V., Brugarolas, J., and Zhang, X. (2010). Structural analysis and functional implications of the negative MTORC1 regulator REDD1. Biochemistry (Mosc.) *49*, 2491–2501.

Venkatesan, A., Sharma, R., and Dasgupta, A. (2003). Cell cycle regulation of hepatitis C and encephalomyocarditis virus internal ribosome entry site-mediated translation in human embryonic kidney 293 cells. Virus Res. *94*, 85–95.

Venot, C., Maratrat, M., Sierra, V., Conseiller, E., and Debussche, L. (1999). Definition of a p53 transactivation function-deficient mutant and characterization of two independent p53 transactivation subdomains. Oncogene *18*, 2405–2410.

Vézina, C., Kudelski, A., and Sehgal, S.N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J. Antibiot. (Tokyo) *28*, 721–726.

Volpon, L., Osborne, M.J., Topisirovic, I., Siddiqui, N., and Borden, K.L. (2006). Cap-free structure of eIF4E suggests a basis for conformational regulation by its ligands. EMBO J *25*, 5138–5149.

Vopalensky, V., Masek, T., Horvath, O., Vicenova, B., Mokrejs, M., and Pospisek, M. (2008). Firefly luciferase gene contains a cryptic promoter. RNA *14*, 1720–1729.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413–431.

Wallander, M.L., Leibold, E.A., and Eisenstein, R.S. (2006). Molecular control of vertebrate iron homeostasis by iron regulatory proteins. Biochim. Biophys. Acta BBA - Mol. Cell Res. *1763*, 668–689.

Wang, G.L., Jiang, B.-H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc. Natl. Acad. Sci. *92*, 5510–5514.

Wang, C., Norton, J.T., Ghosh, S., Kim, J., Fushimi, K., Wu, J.Y., Stack, M.S., and Huang, S. (2008). Polypyrimidine tract-binding protein (PTB) differentially affects malignancy in a cell line-dependent manner. J. Biol. Chem. *283*, 20277–20287.

Wang, L., Harris, T.E., Roth, R.A., and Lawrence, J.C., Jr (2007). PRAS40 regulates MTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. J. Biol. Chem. *282*, 20036–20044.

Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309–314.

Warnakulasuriyarachchi, D., Cerquozzi, S., Cheung, H.H., and Holcík, M. (2004). Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible internal ribosome entry site element. J. Biol. Chem. *279*, 17148–17157.

Weinberg, R.L., Veprintsev, D.B., Bycroft, M., and Fersht, A.R. (2005). Comparative binding of p53 to its promoter and DNA recognition elements. J. Mol. Biol. *348*, 589–596.

Weingarten-Gabbay, S., Khan, D., Liberman, N., Yoffe, Y., Bialik, S., Das, S., Oren, M., and Kimchi, A. (2013). The translation initiation factor DAP5 promotes IRES-driven translation of p53 mRNA. Oncogene.

Weinlich, S., Huttelmaier, S., Schierhorn, A., Behrens, S.E., Ostareck-Lederer, A., and Ostareck, D.H. (2009). IGF2BP1 enhances HCV IRES-mediated translation initiation via the 3'UTR. RNA *15*, 1528–1542.

Wek, R.C., Jiang, H.-Y., and Anthony, T.G. (2006). Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. *34*, 7–11.

Wells, S.E., Hillner, P.E., Vale, R.D., and Sachs, A.B. (1998). Circularization of mRNA by eukaryotic translation initiation factors. Mol. Cell *2*, 135–140.

Wendel, H.-G., Silva, R.L.A., Malina, A., Mills, J.R., Zhu, H., Ueda, T., Watanabe-Fukunaga, R., Fukunaga, R., Teruya-Feldstein, J., Pelletier, J., et al. (2007). Dissecting eIF4E action in tumorigenesis. Genes Dev. *21*, 3232–3237.

Wilker, E.W., van Vugt, M.A.T.M., Artim, S.C., Huang, P.H., Petersen, C.P., Reinhardt, H.C., Feng, Y., Sharp, P.A., Sonenberg, N., White, F.M., et al. (2007). 14-3-3[sgr] controls mitotic translation to facilitate cytokinesis. Nature *446*, 329–332.

Willis, A.E. (1999). Translational control of growth factor and proto-oncogene expression. The International Journal of Biochemistry & Cell Biology *31*, 73–86.

Wilson, J.E., Pestova, T.V., Hellen, C.U.T., and Sarnow, P. (2000a). Initiation of Protein Synthesis from the A Site of the Ribosome. Cell *102*, 511–520.

Wilson, K.F., Wu, W.J., and Cerione, R.A. (2000b). Cdc42 Stimulates RNA Splicing via the S6 Kinase and a Novel S6 Kinase Target, the Nuclear Cap-binding Complex. J. Biol. Chem. *275*, 37307–37310.

Wong, W.L., Brostrom, M.A., Kuznetsov, G., Gmitter-Yellen, D., and Brostrom, C.O. (1993). Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells. Biochem J *289*, 71–79.

Woolaway, K.E., Lazaridis, K., Belsham, G.J., Carter, M.J., and Roberts, L.O. (2001). The 5' untranslated region of Rhopalosiphum padi virus contains an internal ribosome entry site which functions efficiently in mammalian, plant, and insect translation systems. J. Virol. *75*, 10244–10249.

Wouters, B.G., and Koritzinsky, M. (2008). Hypoxia signalling through MTOR and the unfolded protein response in cancer. Nat Rev Cancer *8*, 851–864.

Wu, G.S., Burns, T.F., McDonald, E.R., 3rd, Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., et al. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat. Genet. *17*, 141–143.

Yamagiwa, Y., Marienfeld, C., Meng, F., Holcik, M., and Patel, T. (2004). Translational regulation of x-linked inhibitor of apoptosis protein by interleukin-6: a novel mechanism of tumor cell survival. Cancer Res. *64*, 1293–1298.

Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., and Mori, K. (2007). Transcriptional Induction of Mammalian ER Quality Control Proteins Is Mediated by Single or Combined Action of ATF6α and XBP1. Dev. Cell *13*, 365–376.

Yaman, Ibrahim. "Mechanisms of Post-transcriptional Regulation of Cat-1 Gene Expression by Amino Acid Starvation." Electronic Thesis or Dissertation. Case Western Reserve University, 2005. https://etd.ohiolink.edu/

Yamanaka, S., Poksay, K.S., Arnold, K.S., and Innerarity, T.L. (1997). A novel translational repressor mRNA is edited extensively in livers containing tumors

caused by the transgene expression of the apoB mRNA-editing enzyme. Genes Dev. *11*, 321–333.

Yamazaki, H., Hiramatsu, N., Hayakawa, K., Tagawa, Y., Okamura, M., Ogata, R., Huang, T., Nakajima, S., Yao, J., Paton, A.W., et al. (2009). Activation of the Akt-NF-kappaB pathway by subtilase cytotoxin through the ATF6 branch of the unfolded protein response. J. Immunol. Baltim. Md 1950 *183*, 1480–1487.

Yanagiya, A., Svitkin, Y.V., Shibata, S., Mikami, S., Imataka, H., and Sonenberg, N. (2008). Requirement of RNA Binding of Mammalian Eukaryotic Translation Initiation Factor 4GI (eIF4GI) for Efficient Interaction of eIF4E with the mRNA Cap. Mol. Cell. Biol. *29*, 1661–1669.

Yang, D.Q., Halaby, M.J., and Zhang, Y. (2006). The identification of an internal ribosomal entry site in the 5′-untranslated region of p53 mRNA provides a novel mechanism for the regulation of its translation following DNA damage. Oncogene *25*, 4613–4619.

Yang, H., Rudge, D.G., Koos, J.D., Vaidialingam, B., Yang, H.J., and Pavletich, N.P. (2013). MTOR kinase structure, mechanism and regulation. Nature *497*, 217–223.

Yang, H.-S., Jansen, A.P., Komar, A.A., Zheng, X., Merrick, W.C., Costes, S., Lockett, S.J., Sonenberg, N., and Colburn, N.H. (2003). The Transformation Suppressor Pdcd4 Is a Novel Eukaryotic Translation Initiation Factor 4A Binding Protein That Inhibits Translation. Mol. Cell. Biol. *23*, 26–37.

Yang, Y.L., Reis, L.F., Pavlovic, J., Aguzzi, A., Schäfer, R., Kumar, A., Williams, B.R., Aguet, M., and Weissmann, C. (1995). Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J. *14*, 6095–6106.

Ye, Q., Cai, W., Zheng, Y., Evers, B.M., and She, Q.-B. (2013). ERK and AKT signaling cooperate to translationally regulate survivin expression for metastatic progression of colorectal cancer. Oncogene.

Yin, X., Fontoura, B.M.A., Morimoto, T., and Carroll, R.B. (2003). Cytoplasmic complex of p53 and eEF2. J. Cell. Physiol. *196*, 474–482.

Yin, Y., Stephen, C.W., Luciani, M.G., and Fåhraeus, R. (2002). p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. Nat. Cell Biol. *4*, 462–467.

Yoon, A., Peng, G., Brandenburger, Y., Brandenburg, Y., Zollo, O., Xu, W., Rego, E., and Ruggero, D. (2006). Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. Science *312*, 902–906.

Yoshizawa, T., Hinoi, E., Jung, D.Y., Kajimura, D., Ferron, M., Seo, J., Graff, J.M., Kim, J.K., and Karsenty, G. (2009). The transcription factor ATF4 regulates glucose metabolism in mice through its expression in osteoblasts. J. Clin. Invest. *119*, 2807–2817.

Yu, H., Isken, O., Grassmann, C.W., and Behrens, S.-E. (2000). A stem-loop motif formed by the immediate 5′ terminus of the bovine viral diarrhea virus genome modulates translation as well as replication of the viral RNA. J. Virol. *74*, 5825–5835.

Yu, Y., Yoon, S.-O., Poulogiannis, G., Yang, Q., Ma, X.M., Villén, J., Kubica, N., Hoffman, G.R., Cantley, L.C., Gygi, S.P., et al. (2011). Phosphoproteomic analysis identifies Grb10 as an MTORC1 substrate that negatively regulates insulin signaling. Science *332*, 1322–1326.

Yuan, X.-W., Wang, D.-M., Hu, Y., Tang, Y.-N., Shi, W.-W., Guo, X.-J., and Song, J.-G. (2013). Hepatocyte Nuclear Factor 6 suppresses the migration and invasive

growth of lung cancer cells through p53 and the inhibition of epithelial-mesenchymal transition. J. Biol. Chem.

Xia, X., and Holcik, M. (2009). Strong Eukaryotic IRESs Have Weak Secondary Structure. PLoS ONE *4*, e4136.

Zhai, H., Acharya, S., Gravanis, I., Mehmood, S., Seidman, R.J., Shroyer, K.R., Hajjar, K.A., and Tsirka, S.E. (2011). Annexin A2 Promotes Glioma Cell Invasion and Tumor Progression. J. Neurosci. *31*, 14346–14360.

Zhan, Q., Antinore, M.J., Wang, X.W., Carrier, F., Smith, M.L., Harris, C.C., and Fornace, A.J., Jr (1999). Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene *18*, 2892–2900.

Zheng, G., Dahl, J.A., Niu, Y., Fu, Y., Klungland, A., Yang, Y.-G., and He, C. (2013). Sprouts of RNA epigenetics: The discovery of mammalian RNA demethylases. RNA Biol. *10*, 915–918.

Zheng, X., and Bevilacqua, P.C. (2004). Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. RNA *10*, 1934–1945.

Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M.M., Simons, J.W., and Semenza, G.L. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res. *60*, 1541–1545.

Zhou, D., Palam, L.R., Jiang, L., Narasimhan, J., Staschke, K.A., and Wek, R.C. (2008). Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. J. Biol. Chem. *283*, 7064–7073.

Zilfou, J.T., and Lowe, S.W. (2009). Tumor Suppressive Functions of p53. Cold Spring Harb. Perspect. Biol. *1*, a001883–a001883.

Zinzalla, V., Stracka, D., Oppliger, W., and Hall, M.N. (2011). Activation of MTORC2 by association with the ribosome. Cell *144*, 757–768.

Zoll, W.L., Horton, L.E., Komar, A.A., Hensold, J.O., and Merrick, W.C. (2002). Characterization of mammalian eIF2A and identification of the yeast homolog. J. Biol. Chem. *277*, 37079–37087.

Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., and Sabatini, D.M. (2011). MTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science *334*, 678–683.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. *31*, 3406–3415.